# A NEW RESORBABLE WRAP-AROUND IMPLANT AS AN ALTERNATIVE NERVE REPAIR TECHNIQUE

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**Poly-3-hydroxybutyrate (PHB), a bacterial storage product, is available as bioabsorbable sheets and has been used in this study for primary nerve repair. The aim was to assess axonal regeneration following such repair and determine the inflammatory response to PHB.**

**In 20 adult cats, the transected superficial radial nerve was wrapped in PHB sheets, while primary epineural repair was carried out in the contralateral limb. At 6 and 12 months, the repair sites were assessed immunohistochemically for macrophage infiltration and myelinated axons were counted in the distal nerve.**

**Mean macrophage counts across the whole width of the nerve in both groups at 6 and 12 months showed no statistically significant difference. Nor was there any significant difference between the two groups at both time-points in axon counts, axon diameter, myelin thickness and *g*-ratio. There was a statistically significant increase in fibre diameters at 12 months, indicating that fibres were undergoing continuous maturation.**

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## INTRODUCTION

Current microsurgical techniques for peripheral nerve repair give functional results that are not always optimal (Terzis, 1990). The need to improve these results has led to the quest for a sutureless method of nerve repair that would cause minimal interference with the internal environment of the injured nerve. The use of adhesives such as cyanoacrylate glue (Ferlic and Goldner, 1965) and fibrin (Moy et al., 1988) have not improved results. Welding tissue with carbon dioxide lasers causes thermal damage to the nerve, which exceeds any benefit gained by the absence of foreign material (Richmond, 1986).

Nerve tubulization offers an alternative method of repair of severed nerves with maximal coaptation and minimal injury. Tubulization has several advantages, as it protects regenerating fibres by reducing invasion and scarring of the nerve, and discourages the formation of neuromas.

Microorganisms are capable of producing a wide range of polymers generated from 3-hydroxypropionic acid, which is widely present in nature. One such homopolymer, poly-3-hydroxbutyrate (PHB), is a natural storage product of bacteria and algae and occurs as discrete granules within the cell cytoplasm. PHB granules are produced from bacterial cultures by fermentation followed by solvent extraction. PHB can also be produced from carbon substrates as diverse as glucose, ethanol, acetane, methane and gaseous mixtures of carbon dioxide and oxygen (Anderson and Dawes, 1990). PHB (Astra Tech, Gothenberg, Sweden) is available in the form of sheets which have been used experimentally in cardiovascular surgery (Duvernoy et al., 1995; Malm et al., 1992; 1994). PHB is non-antigenic, biocompatible, easy to handle, has good tensile strength and is completely resorbed within 24 to 30 months by hydrolytic degradation (Gogolewski et al., 1993; Holmes, 1988; Malm et al., 1992; 1994).

The fibres in a sheet of PHB are orientated in one direction which, when a sheet is rolled into a tube, can be orientated along the longitudinal axis. Based on previous experience (Curtis and Wilkinson, 1997; Whitworth et al., 1995), we hypothesized that longitudinally placed fibres would aid neuronal and glial cell growth by contact guidance and mechanical orientation. Hence, Schwann cells would align themselves along the longitudinal fibres, preferentially propagate in the longitudinal axis, and facilitate the regrowth of axons through the conduit. The aim of this study was to test this material for coapting the severed ends of a nerve, providing an end-to-end primary repair without the need for epineural sutures. The extent of the axonal regeneration and the inflammatory response to PHB were assessed morphologically up to 12 months post-operatively, and compared to standard epineural nerve repair.

## MATERIALS AND METHODS

### Surgical procedure

Twenty adult female cats, aged approximately 1 year, weighing 2.6 to 3.7 kg were used in the study. The animals were anaesthetised by subcutaneous medetomidin (0.1 mg/kg), intubated and ventilated with isoflurane/ $O_2$ inhalation. Surgery was carried out under aseptic conditions and continuous monitoring. Under an operating microscope, the superficial radial nerve was exposed bilaterally on the mid-foreleg and transected. On one side, the transected nerve was enclosed in a conduit consisting of a PHB sheet wrapped around the nerve ends, leaving a gap of about 2 to 3 mm between them. The

Fig 1 Intraoperative photograph of PHB wrap-around in situ.

PHB conduits were formed from sheets with longitudinal fibre orientation. The conduits were soaked in tissue fluids in the wound for at least 5 minutes to improve their flexibility.

The formed tube was secured by one 9/0 suture at each end and sealed lengthways with Tisseel glue (Immuno AG, Vienna, Austria) (Fig 1). On the contralateral side, the nerve was sutured with 9/0 epineurial sutures. The wound was closed with resorbable 5/0 sutures. Postoperative reversal of medetomidin was achieved by the administration of atipamezol and the cats received buprenorphine as an analgesic for 3 to 5 days. At either 6 or 12 months, groups of ten animals were premedicated with medetomidin, deeply anaesthetized with intravenous thiopental (1.25 mg/kg) and intubated. Under the operating microscope, 10 mm of the superficial radial nerve at the site of the earlier transection was harvested and fixed overnight in 4% paraformaldehyde for macrophage count. Ten milimetres of the regenerated nerve, 5 mm distal to either the PHB conduit or the epineural repair, was fixed in 2.5% glutaraldehyde overnight for myelinated fibre analysis. The specimens were washed twice and then stored in phosphate buffer (0.1 M, pH 7.4) after fixation. Following the same protocol outlined above, the six superficial radial nerves from non-operated cats were used as normal control.

All experimental procedures were performed according to the European Communities Council Directive (86/609/EEC) and were approved by the Regional Committee for Ethics in Animal Experiments in Gothenberg, Sweden.

### Myelinated fibre analysis

Nerve regeneration was assessed in the nerve distal to the repair site by computerized quantification of myelinated axons. The distal nerve segment was post-fixed with 1% osmium tetraoxide (Agar Scientific, Stansted, UK) in 0.1 M phosphate buffer for 1 hour at room temperature, washed with phosphate buffer and dehydrated serially through increasing concentrations of acetone. Infiltration of the specimen was initially carried out overnight with 1:1 acetone: araldite CY212 resin (Agar Scientific, Stansted, UK), followed by two changes of fresh resin and final embedding in araldite CY212 resin. The blocks were polymerized at 60°C for 18 hours. Semithin (1 μm) transverse sections were cut on a Reichert-Jung Ultracut E ultramicrotome, floated onto distilled water, collected on slides and stained with thionine blue and acridine orange to enhance the myelin contrast. Tissue analysis and all subsequent morphometric assessment were performed on coded sections without knowledge of the source. From each nerve sample, four non-adjacent fields (40 × objective) were captured from randomly chosen sections using a video camera connected to the light microscope. The captured image was automatically edited by background subtraction, image enhancement and thresholding. Monochromatic thresholding coloured the myelin red, contrasting it with the background blue and this 'thresholding' value was maintained within a narrow range for all measured sections. The measured parameters for regenerating myelinated axons were fibre diameter, myelin thickness, *g*-ratio and shape factor. The shape factor of a circle is one and measurements approximating this value indicate the regular shape of the axons. The percentage distribution of fibres according to their diameter was also charted for each group.

### Macrophage counts

The repair sites from both groups were fixed overnight in 4% paraformaldehyde. After three daily changes in phosphate buffered saline (PBS) containing 15% sucrose, the specimens were blocked using OCT (Tissue-tek, Sakura, Japan). Serial longitudinal sections (15 μm) of each repair site were collected on slides coated with Vectabond (Vector Laboratories, Peterborough, UK) and allowed to dry for 4 hours at room temperature. For each sample, one slide was stained with haematoxylin and eosin and the remainder were immunostained by the indirect avidin-biotin complex peroxidase nickel enhancement procedure (Shu et al., 1988) using a monoclonal antibody to ED-1 (Serotec, Kidlington, UK, 1:600 dilution) as a marker specific for macrophages. Before a manual macrophage count, the midpoint of the repair site was identified macroscopically by bisecting the length of the section. Microscopically, in H & E and ED-1 stained sections, the exact location of the junction in both primary and PHB repairs was clearly identifiable by the presence of randomly orientated regenerating fibres and occasionally by the presence of suture holes, co-relating well to the midpoint of the repair identified macroscopically. On two randomly chosen sections from each sample, macrophages were counted in two adjacent

Table 1—Myelinated fibre analysis: results at 6 months. All values are given as mean (SEM). *g*-ratio = axon diameter/fibre diameter

| | *PHB* | *Epineural repair* |
|---|---|---|
| Mean axon count | 971.7 (46.61) | 1029.3 (66.51) |
| Mean fibre diameter (μm) | 5.34 (0.020) | 5.30 (0.018) |
| Mean myelin thickness (μm) | 1.55 (0.004) | 1.51 (0.003) |
| Mean *g*-ratio | 0.41 (0.002) | 0.42 (0.002) |
| Mean shape factor | 0.85 (0.0009) | 0.83 (0.0008) |

fields on either side of the midpoint across the whole width of the nerve (20 × magnification).

## RESULTS

### Myelinated fibre analysis

On qualitative assessment, all specimens demonstrated a mixture of regenerating axons of varying diameters. Degenerating axons, present in minimal numbers, were not counted. Tables 1 and 2 summarize the values at 6 months, 12 months and in normal non-operated nerves respectively. Statistical analysis, using the *t*-test, showed no significant difference for any measured value between the PHB and primary epineural repair groups within each time-point. One way ANOVA comparing PHB wrap-around, epineural repair and normal nerves at 12 months demonstrated a significant difference for all parameters between the PHB group and normal nerves, and similarly between epineural repair and normal groups ($P<0.05$). Comparison within the same experimental group through time (i.e. PHB or primary epineural repair at 6 and 12 months) showed a significantly greater fibre diameter in both the primary repair group (Mann-Whitney rank sum test, $P=0.003$) and the PHB group (Mann–Whitney rank sum test, $P=0.011$) (Table 3). This increase in fibre diameter with time indicates a continuing maturation of the axon. This is more clearly shown in Figures 2 and 3, where the percentage distribution of myelinated axons has been plotted according to their sizes at both time points and in normal non-operated nerves. At 6 months, the percentage pattern in PHB is similar to that of primary repair, both groups showing a unimodal distribution and a preponderance of smaller fibres with a major peak at 3 to 4 μm fibre diameter. At 12 months, the percentage pattern is again similar between the two groups. However, despite a persistent but smaller peak at 3 to 4 μm, the fibre distribution is much wider, with a definite increase in larger diameter fibres (8–12 μm diameter). The normal uninjured nerves exhibited a bimodal distribution over a range of 2 to 15 μm with peaks at 5 and 11 μm. Both operated groups also showed a decrease in mean axon count at 12 months compared with 6 months, which may indicate a die-back of axonal sprouting with continuing maturation.

### Macrophage counts at the repair site

On qualitative microscopic assessment, in the PHB group the polymer remnants appeared to be decreased at 12 months compared with 6 months with disruption in fibre continuity and increased fragmentation of the polymer.

Table 4 summarizes the values of macrophage counts in the epineural repair and PHB groups at 6 and 12 months. Although there was some difference between the groups, this was not statistically significant, indicating that the foreign body reaction to PHB is comparable to that of epineural repair.

## DISCUSSION

The results of this study demonstrate that the axonal regeneration, assessed by myelinated axon counts, was similar in the two experimental groups and that PHB produced an inflammatory response, gauged by the macrophage infiltration, similar to that found in primary epineural repair with no evidence of scar tissue. The conclusion is that PHB wrap-around is a good alternative to epineural repair.

Tubulization is a more "biological" approach to nerve repair whereby the neural tissue is allowed to heal by its intrinsic capacity in a closed space with minimal surgical trauma. Encasing the ends of a transected nerve in a tube with a short gap between the nerve stumps allows accumulation of locally produced neurotrophic factors. In a series of 18 patients, comparing conventional microsurgical repair with silicone tubulization in median and

Table 2—Myelinated fibre analysis: results at 12 months and in normal non-operated superficial radial nerve. All values are given as mean (SEM). $P<0.05$ for all measured parameters on comparison of epineural repair and PHB groups to normal group, one-way ANOVA test, all pairwise multiple comparison procedures (Tukey test)

| | *PHB* | *Epineural repair* | *Normal* |
|---|---|---|---|
| Mean axon count | 896 (62.62) | 942.7 (51.41) | 546.6 (26.31) |
| Mean fibre diameter (μm) | 6.27 (0.026) | 6.10 (0.024) | 9.23 (0.145) |
| Mean myelin thickness (μm) | 1.61 (0.004) | 1.55 (0.003) | 2.03 (0.026) |
| Mean *g*-ratio | 0.48 (0.001) | 0.49 (0.001) | 0.54 (0.006) |
| Mean shape factor | 0.83 (0.0009) | 0.84 (0.0008) | 0.83 (0.006) |

**Table 3—Comparison of fibre diameters within each experimental group. All values are given as mean (SEM) in μm**

| | *PHB* | *Epineural repair* |
|---|---|---|
| Mean fibre diameter | | |
| 6 months | 5.34 (0.020) | 5.30 (0.018) |
| 12 months | 6.27 (0.026) | 6.10 (0.024) |
| *P*-value | 0.011 | 0.003 |

**Table 4—Macrophage counts. All values are given as mean (SEM)**

| | *PHB* | *Epineural repair* |
|---|---|---|
| 6 months | 100 (31) | 72 (21) |
| 12 months | 92 (22) | 87 (19) |

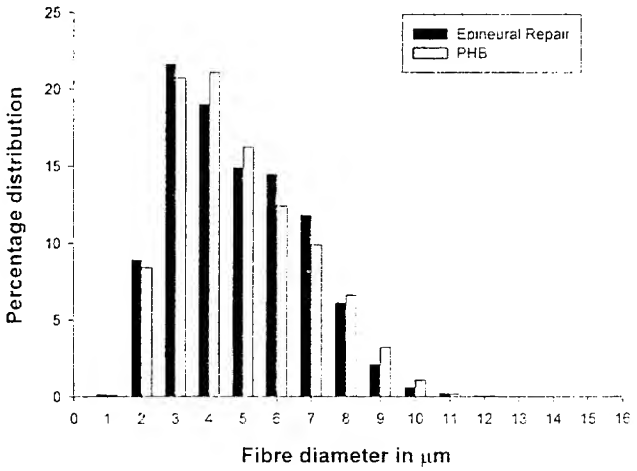


Fig 2 Size distribution of myelinated fibres at 6 months. The percentage pattern of PHB is similar to that of epineural repair, both groups showing a preponderance of smaller fibres, with a major peak at 3 to 4 μm.

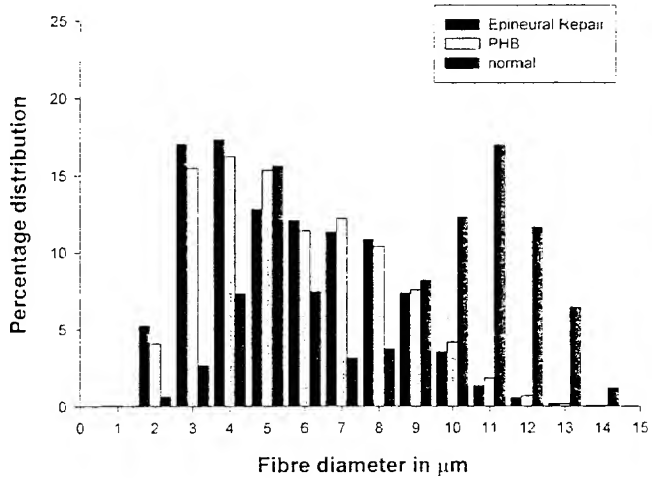


Fig 3 Size distribution of myelinated fibres at 12 months (PHB and epineural repair) and in normal non-operated superficial radial nerves. The percentage pattern in PHB is similar to that of epineural repair. The distribution of both groups is much wider with an increase in larger diameter fibres (8–12 μm). The distribution in normal nerves is bimodal with peaks at 5 and 11 μm.

ulnar nerve injuries, Lundborg et al. (1997) demonstrated no difference in the results between the two techniques. However, a second operation was necessary for removal of the silicone tube as it is non-absorbable and may cause local discomfort. Silicone entubulation can also lead to chronic nerve compression (Merle et al., 1989). As PHB is a bioabsorbable conduit, it would offer an alternative solution for the treatment of nerve injuries. As expected, in this study the polymer underwent progressive degradation, qualitatively observed at 6 and 12 months as disruption in the continuity of the fibres, fragmentation and a decrease in the polymer. The foreign body reaction to PHB, as seen by the macrophage infiltration, was minimal and similar to the reaction to sutures used in epineural repair.

Morphometric analysis of the myelinated axons within the same experimental groups showed significant differences in the fibre diameters at 12 months in comparison to 6 months, with a progressive increase in fibre diameters indicating continuing axonal maturation. The normal non-operated nerves exhibited fibres which were significantly larger at 12 months than in either of the two operated groups, implying that axonal maturation was still ongoing. However, in both PHB and epineural repair groups the axon counts at 6 months were greater than at 12 months. It is known that during regeneration after division, there is sprouting of axon collaterals with subsequent pruning back, most likely as a result of successful connections with the target organs by surviving axons (Brushart, 1993; Lundborg et al., 1994). The myelinated axon counts in normal uninjured nerves were significantly less than either the PHB or epineural repair groups at 12 months, indicating that further connections with target organs and axonal die-back continues to take place at 12 months. The size distribution pattern showed a consistent shift towards an increase in larger axons over time, with a decrease of smaller, possibly branching, fibres. A similar pattern was seen in other studies in which a synthetic conduit was used to bridge a nerve gap (den Dunnen et al., 1993; Dellon and Mackinnon, 1988).

There is a need to improve the functional results obtained from conventional microsurgical repair. Tubulization with a synthetic, bioabsorbable and relatively inert conduit such as PHB offers an alternative solution. It is easier and simpler to encase crushed, transected nerve stumps in a PHB tube, allowing the nerve to heal by its intrinsic capacity, rather than attempt to align the traumatized fascicles correctly by surgery with the risk of further damage.

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# A resorbable nerve conduit as an alternative to nerve autograft in nerve gap repair

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*SUMMARY.* Poly-3-hydroxybutyrate (PHB) occurs within bacterial cytoplasm as granules and is available as bioabsorbable sheets. Previously, the advantage of PHB in primary repair has been investigated while in this study the same material has been used to bridge an irreducible gap. The aim was to assess the level of regeneration in PHB conduits compared to nerve autografts.

The rat sciatic nerve was exposed, a 10 mm nerve segment was resected and bridged with either an autologous nerve graft or a PHB conduit. The grafted segments were harvested up to 30 days. Immunohistochemical staining was performed and computerised quantification of penetration distance and volume of axonal regeneration was estimated by protein gene product (PGP) immunostaining and calcitonin gene-related peptide (CGRP) positive fibres. Penetration and proliferation density of Schwann cells into the conduit was measured by quantifying S-100 staining. The inflammatory response was quantified with ED-1 staining for macrophages. Antibodies to vWf provided an assessment of angiogenesis and capillary infiltration.

Percentage immunostaining for PGP in autograft and PHB groups showed a progressive increase up to 30 days with a significant linear trend with time and an increase in the volume of axonal regeneration. A similar pattern of progressive increase with time was observed with CGRP immunostaining for both groups and with S-100 in the PHB group. Good angiogenesis was present at the nerve ends and through the walls of the conduit. The results demonstrate good nerve regeneration in PHB conduits in comparison with nerve grafts. © 1999 The British Association of Plastic Surgeons

**Keywords:** resorbable nerve conduit, nerve autograft, rat sciatic nerve.

The use of nerve grafts to bridge irreducible nerve gaps results in sub-optimal functional results and donor site morbidity. This has led to a revival of interest in the search for a usable nerve conduit, which needs to have several properties. It should be inert, flexible, bioresorbable and inhibitory to pathological processes such as scarring and oedema, but beneficial to processes of healing and regeneration.[1] Besides being bioabsorbable, the conduit should remain in situ without degradation beyond the period of time it takes the regenerating axons to cross the gap and penetrate the distal stump.

Poly-3-hydroxybutyrate (PHB) is a storage product of bacteria, occurring within the cell cytoplasm as granules. It is available as bioabsorbable sheets, which are non-antigenic, easy to handle and have good tensile strength.[2,3] PHB undergoes hydrolytic degradation and is completely absorbed in 24–30 months.[4,5] Recently, the advantages of a PHB wrap-around in primary repair have been demonstrated.[6] In this study, the same material has been used as a conduit to bridge a 10 mm gap in the rat sciatic nerve. The aim was to assess the level of regeneration in a PHB conduit in comparison with the current standard using a nerve autograft.

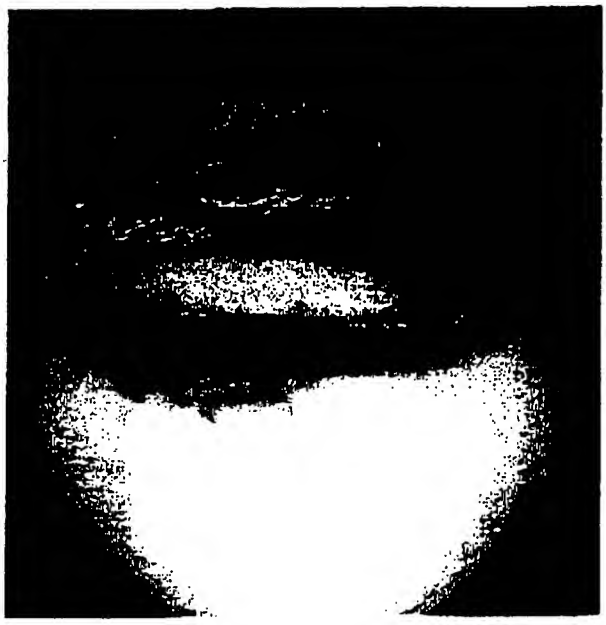

**Figure 1**—Intraoperative photograph of PHB conduit bridging a 10 mm gap in the left sciatic nerve.

## Materials and methods

### *Operative procedure*

Thirty-six 8-week-old inbred male Lewis rats were used in the study. The animals were anaesthetised

This work was presented at the 1998 Summer meeting of the British Association of Plastic Surgeons, Colchester, UK and was awarded the John Calder Prize.

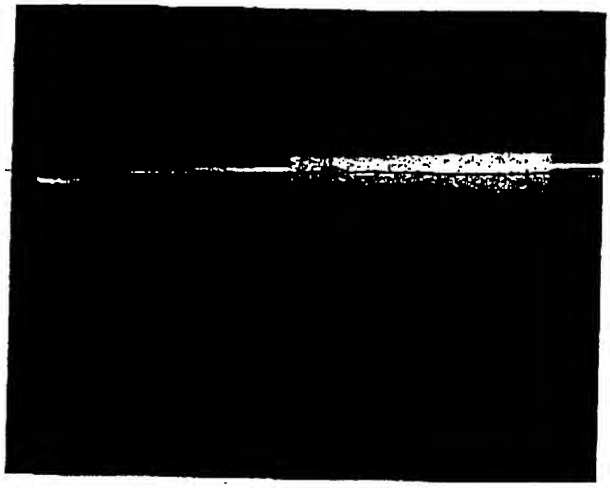

Figure 2—Unidirectional fibre orientated sheet of PHB (8 × 14 mm) rolled around a 16 G cannula to form a tube 14 mm long and with an internal diameter of 1.6 mm.

Figure 3—Intraoperative photograph of a reversed sciatic nerve autograft.

with 0.3 ml/kg of intramuscular Hypnorm (Fentanyl citrate, 0.315 mg/ml; fluanisone, 10 mg/ml; Janssen Cilag, High Wycombe, UK) and 2.5 mg/kg of intraperitoneal diazepam (Phoenix Pharmaceuticals, Gloucester, UK). All procedures were carried out in compliance with UK Home Office regulations.

Under an operating microscope (Wild, Heerbrugg, Germany), the left sciatic nerve was exposed via a gluteal muscle splitting incision. In the PHB group, a 5 mm nerve segment was resected to produce a 10 mm nerve gap after retraction of the nerve transected ends. The gap was bridged using a 14 mm PHB conduit, entubulating 2 mm of the nerve stump at each end (Fig. 1). Two 10/0 nylon sutures (Ethilon, Ethicon, Edinburgh, UK) were used to anchor the conduit to the epineurium at each end.

The conduits were formed from PHB sheets (5 × 10 cm PHB patch, Astra Tech, Gothenberg, Sweden). Rectangular pieces measuring 8 × 14 mm were cut, ensuring that the orientation of the PHB polymer fibres was along the longitudinal axis as previous experiments have shown that longitudinal orientation of PHB fibres promotes neuronal and glial growth by contact guidance. The sheets were then rolled around a 16G intravenous cannula (16G Abbocath®-T. Abbott Ireland, Sligo, Republic of Ireland), thus standardising the internal diameter of the conduits at 1.6 mm (Fig. 2), leaving adequate space for post-injury swelling of the 1 mm-diameter rat sciatic nerve. The rolled sheets were sealed longitudinally with cyanoacrylate glue (Histoacryl®, Braun Melsungen AG, Melsungen, Germany). The conduits, still rolled around the template, were pre-soaked in normal saline to saturate the polymer and ensure maximum expansion of the fibres without a reduction in the internal diameter of the conduit.

In the autograft group, a 10 mm nerve segment was resected, reversed and re-sutured in the gap with 10/0 nylon sutures (Fig. 3). Tension was avoided and atraumatic handling and correct rotational alignment were employed throughout all procedures.

Six animals from each group were sacrificed at 7, 14 and 30 days postoperatively. The repair sites (PHB conduit or nerve autograft) with a 2 mm length of proximal and distal nerve were harvested en bloc, pinned onto a card to avoid shrinkage and marked at the proximal end. Fixation was carried out in Zamboni's solution for 6 h at room temperature and then washed three times with phosphate buffered saline (PBS) containing 15% sucrose and 0.1% sodium azide.

*Immunohistochemistry*

The specimens were blocked in OCT compound (Tissue-tek, Sakura, Japan). The orientation of each specimen was maintained by placing a piece of rat liver next to the proximal end. Serial longitudinal sections (15 μm) were collected on slides coated with Vectabond (Vector Laboratories, Peterborough, UK) and air-dried for 4 h at room temperature. Immunohistochemical staining was carried out using the indirect avidin–biotin complex peroxidase nickel enhancement method.[7] A panel of antibody markers was employed comprising of protein gene product 9.5 (PGP 9.5, Affiniti, Mamhead, UK; dilution 1:5000) a pan-neuronal marker; calcitonin gene-related peptide (CGRP, Affiniti, Mamhead, UK; dilution 1:8000) a marker of sensory nerve fibres; S-100 (Dako A/S, Glostrup, Denmark; dilution 1:8000) an antibody marker for Schwann cells; ED-1 (Serotec Ltd, Kidlington, UK; dilution 1:600) for macrophages; and finally Von Willebrand Factor (vWf, Dako A/S, Glostrup, Denmark; dilution 1:5000) for endothelial cells.

Tissue analysis and all subsequent morphometric assessments were performed on coded sections according to a published protocol[8,9] without the knowledge of the source. A computerised image analysis system (Seescan Analytical Services, Cambridge, UK) was used throughout the study. The volume of regeneration was assessed by capturing images (magnification × 25) from two random sections, at a fixed distance of

Table 1 Maximum regeneration distance from the proximal anastomosis. Distances are given in millimetres as the mean (SEM), $n$=6

| | | Day 7 | Day 14 | Day 30 |
|---|---|---|---|---|
| PGP | AG | 7.62 (0.57)* | >10* | >10 |
| | PHB | 1.02 (0.18) | 4.35 (0.37) | >10 |
| CGRP | AG | 8.15 (0.28)* | >10* | >10 |
| | PHB | 0.79 (0.17) | 3.56 (0.49) | >10 |

* $P$ <0.001, AG vs PHB at 7–14 days; AG = nerve autograft; PHB = conduit.

Figure 4—PGP immunostained regenerating axonal front in a PHB conduit at 7 days (× 20 objective magnification).

2 mm from the proximal repair site across the whole width of the conduit/graft, using a video camera connected to a light microscope. The captured image was automatically edited by background subtraction, image enhancement and thresholding within a narrow range for all measured frames.

The percentage of immunostaining for PGP, CGRP and S-100 between the autograft and PHB groups was then compared at the various time-points, as an indication of the number of regenerating fibres and Schwann cells. Schwann cell proliferation density distance was measured only in the PHB group, as in the autograft group, measurement of S-100 was not possible due to the inherent Schwann cell population. The penetration distance, indicative of the rate of regeneration of PGP and CGRP immunostained fibres, was measured from the proximal anastomosis (magnification × 10) in the autografts and from the beginning of the PHB polymer in the conduits. A correction factor of '−2 mm' was subtracted from all values obtained in the PHB conduit group to compensate for the entubulation of 2 mm of the proximal nerve stump into the conduit.

The inflammatory reaction was measured in both groups by manual counting of macrophages at the midpoint of the graft/conduit across the whole width of the nerve (magnification × 20) in two randomly chosen sections stained with ED-1. VWf staining was used to make a qualitative assessment of the angiogenesis and capillary infiltration.

Table 2 Percentage of immunostaining per frame 2 mm from the proximal anastomosis across the whole width of the nerve. All values are expressed as mean (SEM)

| | | Day 7 | Day 14 | Day 30 |
|---|---|---|---|---|
| PGP | AG | 3.31 (0.81) | 5.84 (0.81) | 7.12 (0.89)* |
| | PHB | 3.59 (0.81) | 3.63 (0.81) | 4.13 (0.81) |
| CGRP | AG | 2.72 (0.61) | 3.19 (0.61) | 5 (0.67) |
| | PHB | 2.86 (0.61) | 2.88 (0.61) | 3.27 (0.61) |
| S-100 | PHB | 5.93 (1.004) | 11.16 (0.48) | 12.06 (1.27) |

*$P$<0.001; $t$-test with Bonferroni correction; PGP at 30 days. AG vs PHB. AG = nerve autograft; PHB = conduit.

## Results

### *Rate of axonal regeneration*

The rate of regeneration was measured as penetration of graft/conduit by the furthermost immunostained fibres from the proximal anastomosis. Table 1 outlines the distance of regeneration for PGP and CGRP immunostained regenerating axons. At 7 days, axons in the nerve graft had crossed two-thirds of the nerve graft and in the PHB conduit had penetrated 1.02 mm of the conduit (Fig. 4). By 14 days, regenerating axons had reached the distal stump in the nerve grafts, whereas these were almost up to the halfway mark in PHB conduits. At 30 days, regenerating fibres in all animals in both groups had reached the distal anastomosis. For PGP immunostained axons, there was a significant difference in the regenerative distance between the two groups along time at 7 and 14 days (two-way ANOVA, $P$<0.001). This pattern was reflected in CGRP immunostained fibres (Table 1).

### *Amount of regeneration*

The values for percentage of immunostaining of PGP and CGRP at 7, 14 and 30 days are given in Table 2. Comparison between the two groups at each of the time-points was not statistically significant except for PGP at 30 days. There was also a linear trend with time for both PGP ($P$=0.01) and CGRP ($P$=0.04) (Fig. 5), indicating that though axonal regeneration identified with the two markers is comparable between the graft types and shows a progressive increase with time, the level of regeneration in PHB conduit is slower, the difference being significant by 30 days.

Schwann cell proliferation density measured in the PHB group showed an increase with time, as shown in Table 2. Though there was a linear trend with time, the Schwann cell density appeared to reach a plateau between 14 and 30 days (Fig. 6). At this time, the axons had reached the distal stump and the levelling effect may be a reflection of the decreased impetus for Schwann cells to proliferate.

### *Macrophage infiltration*

The inflammatory reaction to PHB was gauged by macrophage infiltration of the conduit. ED-1 immunostained macrophage counts for both groups

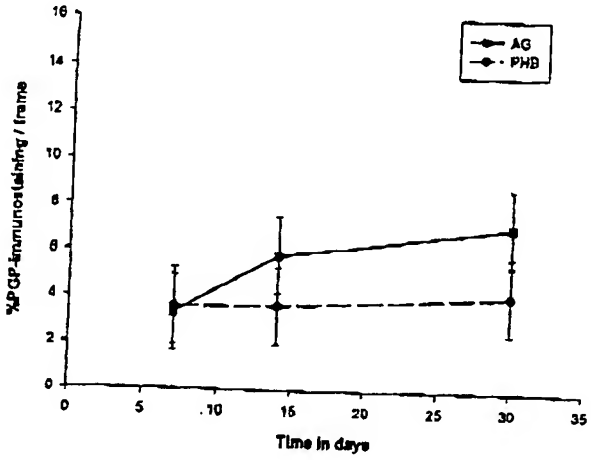


Figure 5—Amount of axonal regeneration, PHB vs nerve autograft at 7, 14 and 30 days, showing significant linear trend. $P<0.05$, two-way ANOVA.

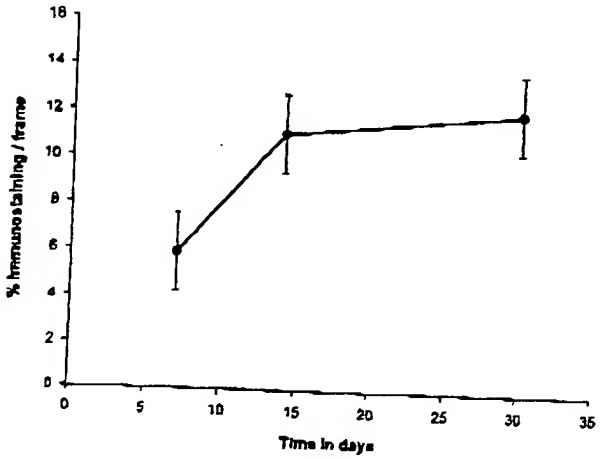


Figure 6—Schwann cell proliferation density in PHB conduit at 7, 14 and 30 days showing significant linear trend. $P<0.001$, one-way ANOVA.

are summarised in Table 3. Comparison between autograft and PHB groups showed no significant difference for macrophage counts at any of the time-points and demonstrates that the inflammatory response to PHB is not intense and was similar to that seen in nerve grafts. On the other hand, an interesting observation is that macrophage numbers appeared to increase from 7 to 14 days and then decreased to their lowest at 30 days. A two-way analysis of variance showed significantly higher numbers in both groups at 14 days compared to 30 days. This correlates well with an infiltration by macrophage scavengers at the injury site following transection, the activity increasing at 14 days and then tailing off as the regenerating axons reach the distal stump by 30 days.

*Angiogenesis*

A qualitative assessment of the vWf immunostained sections showed capillary penetration of the PHB conduit from either end along the proximal and distal nerve stumps and through the walls of the conduit.

Table 3 Macrophage counts. Results of AG and PHB at 7, 14 and 30 days. Macrophages were counted one frame wide at the midpoint of the conduit/graft across the whole width of the nerve at x 20 magnification. All values are given as mean (SEM). Two-way ANOVA, 14 vs 30 days

| | *Day 7* | *Day 14* | *Day 30* |
|---|---|---|---|
| AG | 15 (1.82) | 20 (1.53) | 12 (1.6) |
| PHB | 16 (2.14) | 18 (2.7) | 11 (1.2) |

AG = nerve autograft; PHB = conduit.

## Discussion

The last 20 years have seen the development of many synthetic conduits and several excellent reviews are available summarising the strengths and failings of each of these.[1,10–13] Silicone has been the most widely used for experimental tubulisation and has also been applied clinically.[14] However, due to the lack of degradation of the silicone implants, it has been advocated that the next step in nerve gap repair is the use of a bioabsorbable synthetic conduit[14] which would elicit the most minimal of inflammatory reactions and would remain in situ long enough to support regeneration. Flexibility of the conduit is also necessary to enable continued protection of the regenerating nerve on initiation of mobilisation of the injured part.

PHB fulfils these criteria to a large extent. Besides being bioabsorbable, it elicits a low macrophage reaction comparable to that of a nerve graft. This was also observed in our previous study wherein a PHB wrap-around sheath to join the divided nerve was compared to primary epineural nerve repair.[6] In this study, macrophage numbers in both groups are similar with no significant difference, increasing from 7 to 14 days, this increase correlating well with the period of intense phagocytic activity during Wallerian degeneration[15] and then decreasing significantly by 30 days when the axons have reached the distal stump. Clinically, a low inflammatory response is desirable to prevent adhesions to surrounding structures, in particular to tendons in hand injuries.

The rate of regeneration measured by the regenerative distance into the conduit from the proximal repair site, though not fully equivalent to that of a nerve graft, still appears to be comparable for both PGP and CGRP immunostained fibres. Regenerating axons grow into the first part of the PHB conduit by 7 days, come up to the halfway mark by 14 days and reach the distal stump by 30 days, and it is important to note that there was no failure of regeneration in any of the implanted conduits. This time-scale is in keeping with observations made when other biodegradable conduits such as fibronectin mats have been used.[9] Despite good results obtained with fibronectin mats, the clinical application of this type of conduit may be difficult as it undergoes rapid reabsorption in 3 weeks and has inherent problems associated with pooled plasma from which it is manufactured.[8,9] A longer reabsorption time, as with PHB, ensures that the regenerating and maturing nerve is able to withstand the stress of mobilisation.

The volume of axonal regeneration quantified by the percentage of PGP and CGRP immunostaining appears to be comparable between the two graft types. There is a progressive increase in the amount of regeneration with time, although the difference between nerve grafts and PHB becomes statistically significant by 30 days. The regeneration pattern in the PHB conduit is reflected by the Schwann cell proliferation density, which levels off between 14 and 30 days. This result correlates well with the slowing down of the rate of Schwann cell proliferation demonstrated after the initial burst during Wallerian degeneration, which is thought to be signalled by axonal contact.[16,17]

In conclusion, this study demonstrates good axonal regeneration in PHB conduits with a low level of inflammatory infiltration. This is a good indication of the suitability of PHB as a resorbable synthetic conduit for nerve gap repair. We are aware that the rate and amount of regeneration in PHB conduits does not fully compare with that observed in a nerve graft, but this difference is due to the cellular elements inherently present in nerve grafts which aid regeneration. It is well known that de-differentiated Schwann cells synthesise neurotrophic factors[18] which are known to promote nerve regeneration when administered exogenously to an injured nerve.[8,19] It is tempting to speculate that the level of regeneration in a PHB conduit may be further improved by the addition of growth factors, bringing nearer the concept of a composite conduit to obtain optimal nerve regeneration.

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# NEURONAL SURVIVAL USING A RESORBABLE SYNTHETIC CONDUIT AS AN ALTERNATIVE TO PRIMARY NERVE REPAIR

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Clinically optimal situations for primary nerve repair are rarely observed. Crushed nerve ends result in either suboptimal repair or a need for nerve grafting. Functional results after nerve surgery are relatively poor, including major sensory deficits, which may be due to the death of primary sensory neurons that follows the nerve injury. The aim of this study was to determine if using polyhydroxybutyrate (PHB), a resorbable nerve conduit, could be an alternative to primary nerve repair in reducing loss of neurons. The superficial radial nerves in 20 cats were sectioned bilaterally and primarily repaired microsurgically by the use of two different strategies; either wrapping the nerve ends in sheets of PHB or epineurally suturing of the nerve. After 6 or 12 months, the surviving neurons within the dorsal root ganglia [C5–T1] were counted. No statistically significant differences were found between the two methods. This may imply a future possibility of using PHB as a synthetic nerve graft in situations where suboptimal primary repair or nerve grafts are the alternatives.


MICROSURGERY 19:259–264 1999

Nerve injuries are frequently encountered in the practice of hand surgery. Despite microsurgical repair, they are usually followed by functional loss, especially concerning sensation.[1] This often leaves the patient with a considerable disability. The poor results may be partly due to the pronounced loss of primary sensory neurons located in the dorsal root ganglia (DRG). After axotomy, a change in the phenotype of the neurons will occur[2] and a substantial number of neuronal cells in the DRG will die.[3–6] However, exact microsurgical suturing will only reduce this cell death to a minor extent.[5] Moreover, since peripheral nerve trauma commonly involves crushed ends of the injured nerve, which results in loss of neuronal tissue, clinically optimal situations for primary repair are rarely observed. A shortening of the nerve makes it necessary to make primary repair while it is under tension, which is not satisfactory in most cases.[7] Another alternative is bridging the gap using autologous nerve grafts, which results in morbidity at the donor site.[8] Currently, it is sometimes impossible to harvest enough autologous tissue from the injured patient, especially when the trauma is characterized by extensive defects involving larger nerves. Since the patient may lose sensation and there is a risk of developing neuromas at the graft donor site, several different materials have been scientifically tested as substitutes for nerve grafts. These include silicone tubes, freeze-thawed muscle, vein grafts, and resorbable synthetic and biological materials.[9,10] Unfortunately, the results so far have been in favor of nerve grafts, although silicone tubes and muscle grafts have already been used clinically.[11,12] The idea of repair by entubulation is based on the theory that growth-promoting factors accumulating in the gap between the nerve ends will support axonal regeneration.[13–15]

Poly-β-hydroxybutyrate (PHB) is a biodegradable polymer naturally existing in microorganisms as energy storage granules.[16,17] It can be produced from bacterial cultures[16] and then harvested and processed into materials suitable for different purposes.[16,18–21] In the body, PHB is degraded slowly by hydrolysis and enzymatic influence and will remain there at least 6 months, depending on the PHB surface area in contact with the tissue, the type of tissue, and the species in which it is implanted.[17–21] The degradation product is β-hydroxybutyric acid, a common and nontoxic metabolite in mammals.[17] The present material, produced and provided by Astra Tech (Mölndal, Sweden), is a nonwoven sheet with unidirectional fiber orientation that has previously been clinically studied as a repairing material in cardiovascular surgery.[19,20,22] By examining the neuronal loss within the DRG of a purely sensory nerve, this study aimed to compare nerve repair using PHB conduits in the uncomplicated situation to immediate microsurgical suturing.

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## MATERIALS AND METHODS

### Animals

Twenty adult female cats about 1 year old, weighing 2.6–3.7 kg, were used in the study. The animals were kept together in a large room at the animal department at Astra Hässle (Mölndal, Sweden). All operations were performed in the operating theatres there under aseptic conditions and continuous monitoring. The experiments were approved by the Regional Committee for Ethics in Animal Experiments in Gothenburg and performed according to the European Communities Council Directive (86/609/EEC).

### PHB Conduit

Sterilized PHB sheets of approximately 150 kD molecular weight and unidirectional fiber orientation were processed and provided by Astra Tech AB. Patches were soaked in tissue fluid in the wound for 3–21 minutes until they were flexible enough to form a tube.

### Anesthesia

Anesthesia was induced with 0.15 mg/kg medetomidin subcutaneously (s.c.). The cats were intubated and kept anesthetized on isoflurane/$O_2$ inhalation. To diminish pharyngeal reflexes, lidocaine gel was used at intubation. After surgery, the medetomidin effect was reversed by atipamezol. For infection prophylaxis, the cats received a single dose of 150 mg bensylpenicilline intramuscularly (i.m.). Buprenorphine was given for analgesia for 3–5 days.

### Surgical Procedure

Using the microscope, the superficial radial nerve was exposed bilaterally on the mid-foreleg and transected. In the first group of 10 animals, the nerve was immediately sutured epineurally with 9-0 S&T® (S&T, Switzerland) sutures or it was enclosed in a conduit consisting of a sheet of PHB with its fibers oriented longitudinally, wrapped around the nerve ends, leaving a gap of about 2–3 mm between them. The tube formed was secured to the epineurium by one 9-0 suture in each end and sealed by fibrin glue (Tisseel®, Immuno, Sweden) (Fig. 1). In the second group of 10 animals, a prelabeling of the proximal nerve stump was performed by applying the fluorescent tracer Fast Blue (FB), 2.5% in sterile water, to the proximal nerve end placed in a plastic cup for 2 hours.[23] This was sealed by silicone grease and vaseline in order to prevent leakage. The wound was kept moist during exposure. The nerve was then carefully washed with saline and repaired in either way, i.e., PHB entubulation or primary suture. The wound was in all cases closed with resorbable 5-0 Vicryl® (Ethicon, Germany) sutures.

Both groups of animals were subdivided equally for the two survival times of 6 and 12 months, respectively. After this time, the animals in the first group (i.e., the group not prelabeled) were reanesthetized and the nerve was sectioned

*Figure 1. The superficial radial nerve sectioned and wrapped in a PHB conduit sealed by fibrin glue. Magnification, x6.*

proximal to the first site of injury. This was done to enable the use of another nerve tracer, horseradish peroxidase (HRP).[24] The proximal end was placed in a cup containing 50 µl of 30% HRP in sterile water. After 1 hour, the nerve was removed from the cup and 5 µl of choleratoxin-B-subunit-conjugated HRP (Sigma, St. Louis, MO) was injected into the nerve using a Hamilton syringe. The nerve end was rinsed with saline. To prevent any leakage of HRP to the surrounding tissues, it was wrapped in an envelope of self-adhesive polyurethane (Tegaderm®, Sollentuna, Sweden), which was secured to the epineurium by a few 9-0 sutures. The wound was then closed with resorbable sutures. After another 3 days, the cats were premedicated with medetomidin and deeply anesthetized with tiopental (1.25 mg/kg) intravenously (i.v.). They were intubated and fixed by transcardial perfusion with 4 L of a mixture of 1.5% glutaraldehyde + 1% paraformaldehyde in 0.1 M phosphate buffer (37°C, pH 7.4), preceded by a rinse with 2 L of heparinized buffer. Fixation was terminated with 2 L of cold (4°C) phosphate buffer. The FB-labeled animals (the second group) were perfused with buffer, 4% paraformaldehyde, and without the concluding buffer rinse.

### Tissue Preparation

After fixation, the DRG [C5-T1] from the HRP-labeled animals were removed bilaterally and stored overnight in 30% sucrose. They were then cut into serial 40-µm sections on a freezing microtome and incubated for HRP histochemistry using tetramethylbenzidine.[25] The DRG [C5-T1] from the FB-labeled animals were postfixed in 4% paraformaldehyde overnight, immersed in 20–30% sucrose during the next 2 days for cryoprotection, and frozen at –80°C. Forty-micron sections from a Cryostat microtome were cut and mounted on slides for analysis.

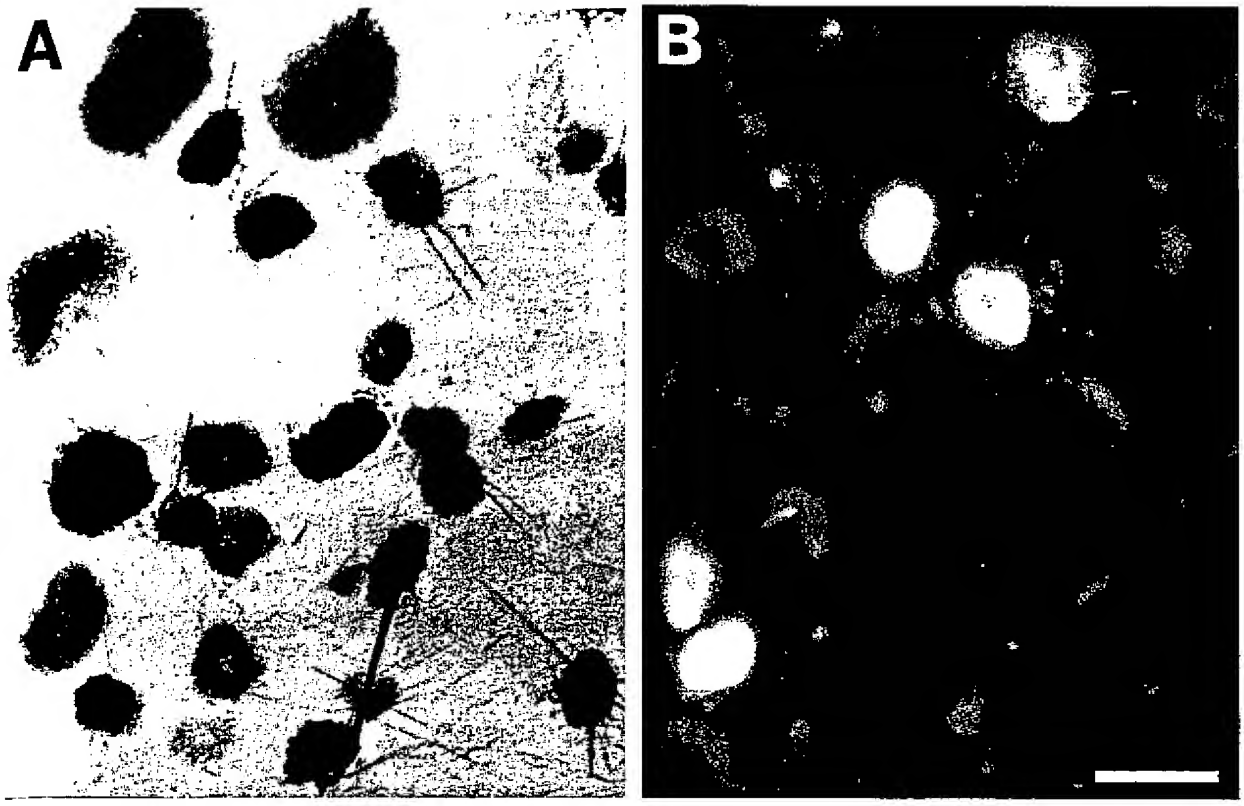


*Figure 2. Photomicrographs at ×250 magnification (scale bar = 100 μm). **A:** HRP-labeled neurons in DRG [C7] 12 months after sectioning of the superficial radial nerve. **B:** FB-labeled neurons in DRG [C8] after 6 months.*

### Cell Number Calculations

In every section, all HRP-labeled neurons were counted using light microscopy at ×160 final magnification (Fig. 2A). The staining was very dense, so neurons had to be counted as soma profiles. Corrections for split cells were made according to Königsmark's formula no. 4.[26] The cell size distribution for small, medium-sized, and large cells found in previous experiments was used,[5] resulting in the total number of cells being multiplied by a factor of 0.574.

FB-labeled neurons were counted in each section as clearly stained, intact nucleated somas under the Leitz (Wetzlar, Germany) Aristoplan fluorescence microscope using filter block A at ×250 final magnification (Fig. 2B). Corrections for split nuclei were made by multiplying the mean cell count by a factor 0.72 according to Abercrombie,[27] after the cell soma size in 100 cells from each group had been measured and the mean diameter calculated using the Eutectic Neuron Tracing System (Eutectic Electronics, Inc., Raleigh, NC). All countings were done by the same person and without information on which side had been sutured or repaired with a PHB conduit.

An overview of the different experiments is given in Table 1. A statistical analysis was performed for each group, comparing the PHB-repaired side with the sutured, using the Wilcoxon signed rank test with the aid of the statistical computer program StatView® (Abacus Concepts, Inc., Berkeley, CA).

**Table 1.** Overview of Experimental Groups and Results

| Cat no. | Tracer | Soaking time (minutes) | Corrected cell no. PHB | Corrected cell no. Suture | Survival (months) |
|---|---|---|---|---|---|
| 1 | HRP | 15 | 6,325 | 5,826 | 12 |
| 2 | HRP | 10 | 8,855 | 6,984 | 12 |
| 3 | HRP | 3 | 8,073 | 8,645 | 12 |
| 4 | HRP | 5 | 7,429 | 5,829 | 12 |
| 5 | HRP | 9 | 5,931 | 6,332 | 12 |
| 6 | HRP | 8 | 1,812 | 2,262 | 6 |
| 7 | HRP | 7 | 2,988 | 1,673 | 6 |
| 8 | HRP | 13 | 3,097 | 1,007 | 6 |
| 9 | FB | 5 | 340 | 269 | 12 |
| 10 | FB | 11 | 184 | 229 | 12 |
| 11 | FB | 13 | 302 | 917 | 12 |
| 12 | FB | 15 | 119 | 434 | 12 |
| 13 | FB | 21 | 905 | 690 | 12 |
| 14 | FB | 8 | 1,009 | 1,292 | 6 |
| 15 | FB | 6 | 597 | 839 | 6 |
| 16 | FB | 14 | 753 | 1,011 | 6 |
| 17 | FB | 6 | 1,575 | 713 | 6 |
| 18 | HRP | 9 | 5,194 | 4,645 | 6 |
| 19 | FB | 6 | 960 | 828 | 6 |
| 20 | HRP | 6 | 4,190 | 4,690 | 6 |

## RESULTS

### General Observations

All the cats tolerated the operations well, although two cats had one wound resutured 10 days postoperativelly due

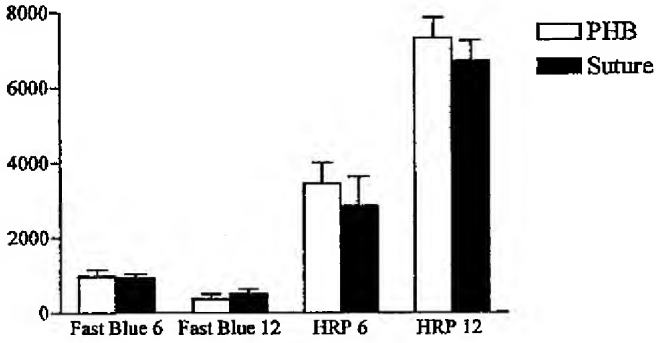


*Figure 3. Diagram showing the mean cell number in DRG ± SEM for the different experimental groups.*

to failed healing (no. 17/PHB side and no. 19/control side, respectively) and one was treated with a single dose of antibiotics due to local postoperative swelling. This was observed 9 days after the sutures had been removed (no. 15/PHB side). At the time of perfusion, the nerves were inspected. In five cats (nos. 8,9,13,14,17), the PHB conduit was suspected to have sprung open, with swelling around the regenerated nerve. There was no correlation between the length of time the sheets had soaked in the tissue fluids and their tendency to spring up. However, it was found that if the PHB soaking time exceeded 5 minutes, the patch became easier to handle when wrapping it around the nerve ends.

There was a trend toward more adhesions to the skin and the radial artery when nerves were sutured than when enclosed by the conduits, particularly for the shorter survival time and in this group predominantly for prelabeled animals, where there was a pronounced neovascularization. PHB tubes were still macroscopically clearly visible at 12 months, but were softer on palpation and easier to dissect from the surrounding tissues than at 6 months.

HRP- as well as FB-labeled neurons were found in DRG [C6-T1], the majority in [C7] and [C8]. There was an interindividual difference in cell numbers, but only in a few cats (nos. 8,11,12,17) were there any marked differences between sides. In one of these (no. 17), there was a correlation to the postoperative problems mentioned above and to peripheral macroscopical findings of poor regeneration, but the sprung up conduits did not cause any corresponding increased cell loss in the DRG (Table 1). The number of cells differed between the two groups of either tracer. This was due to differences in counting technique and the nature of the tracing substances, with corrected cell means for HRP at 2,855 on the control side and 3,456 at the PHB side after 6 months. For FB, these numbers were 937 and 978, respectively. At 12 months, the means were 6,723 and 7,322 in the HRP animals and only 508 and 370 in the FB animals (Fig. 3).

**Table 2.** Descriptive Statistics and Result of Wilcoxon Signed Rank Test

| Experimental group | Mean cell no. ± SEM PHB | Suture | P* |
|---|---|---|---|
| FBlue 6 months | 979 ± 166 | 937 ± 101 | 0.68 |
| FBlue 12 months | 370 ± 140 | 507 ± 131 | 0.5 |
| HRP 6 months | 3,456 ± 575 | 2,855 ± 766 | 0.22 |
| HRP 12 months | 7,323 ± 541 | 6,723 ± 525 | 0.34 |

**Predetermined significance level P = 0.05.*

### Statistical Analysis

The analysis of the number of labeled neurons was performed as a nonparametric, paired test. For all animals, the contralateral side served as a control for the experimental one. The number of labeled DRG cells did not show any statistically significant difference between the nerves that were either repaired with the PHB conduit or primary sutured. This was the case for both the 6- and 12-month survival periods and for both nerve tracers used. The results of the statistical analysis are presented in Table 2.

## DISCUSSION

An evaluation of the resorbable material PHB, being used as a nerve conduit, was performed in this study by comparing it to an optimal situation of direct primary suture. In nerve repair, the idea of using some kind of prosthesis has been an appealing solution especially in nerve defects, in order to avoid the negative consequences of nerve graft harvesting. A nerve conduit with a unidirectional, longitudinal fiber orientation also has the advantage of protecting and mechanically guiding the neurites toward the distal stump even when the nerve ends are lacerated. The fluid forming in its interior contains neurotrophic activity,[28] derived mainly from the degenerating nerve tissue. It gradually becomes a matrix containing fibronectin, laminin, and inflammatory cells, all believed to support the regeneration process.[13,29–33] A number of different materials have been used in attempts to reconstruct divided nerves by entubulation,[9,10] some of them absorbable. A bioabsorbable material will not give rise to a chronic condition of irritation or compression. Hence, removal of the conduit is not required, as seen with silicone tubes.[11,34]

PHB has the advantage of being slowly resorbable in vivo,[17] thereby supporting the nerve during a period long enough for regeneration. The long degradation time also has the benefit of not giving rise to great amounts of acid degradation products that are known to be formed by rapidly resorbable materials.[35] PHB is well tolerated when implanted subcutaneuosly in mice.[21] However, it gives rise to a mild foreign body reaction involving inflammatory cells, mainly macrophages, that gradually decreases to form a fibrous capsule after 6 months.[19–21] The cellular infiltration

is interesting, since macrophages are believed to contribute to regeneration-promoting factors at the site of injury.[29,36] We did find a macroscopically evident inflammation, but this was not more extensive than for the sutured nerves; the nerves reconstructed with PHB had even less adhesions of the conduit to surrounding tissues than sutured ones, meaning that scar tissue will not disturb the regeneration process or impair gliding structures close to the nerve. There was a relation between the degree of inflammation and the FB prelabeling, particularly at 6 months. We believe that the tracer contributed to the increased inflammatory reaction in these cases.

There was an interindividual difference in cell numbers, but only in four cats were there any greater differences between sides. One cat among these (no. 17) had both a short PHB soaking time and a sprung up conduit, as well as postoperative wound rupture and poor macroscopical peripheral regeneration. One (no. 8) had a sprung up conduit, one (no. 11) had poor peripheral regeneration, while in the fourth (no. 12) none of these factors explained the difference between the sides (Table 1). Interestingly enough, nos. 17 and 8 did not have less DRG cells on the side repaired with the PHB conduit.

The method of calculating the number of neurons after application of intraaxonally transported tracers has been questioned for different reasons. HRP is well known as a neuronal tracer, although not always totally reliable, partly due to histochemistry. Earlier investigations have demonstrated a large variation between animals.[5,6] Therefore, in addition to using the contralateral side as a control in every animal, we chose to complement the HRP method with a second tracer method that does not require histochemical processing and that has a nontoxic nature. FB has been used for some time and is found to have similar labeling qualities as HRP.[37,38] It has been found to be transported over long distances,[39] which is of importance in this experiment. Another advantage using FB is the prelabeling technique, which avoids additional surgical exposure that might affect the intraaxonal transport capacity of the nerve. However, there were differences in cell numbers between the methods (Table 1). Since the FB-labeled cells are counted strictly as nuclei in intact cells, the error due to split objects will be less than when counting HRP-labeled cells, even after correction of the latter number as these are counted as soma profiles. There have also been results showing that FB might not label as great a neuronal pool as HRP.[40]

The difference between the survival periods in FB animals is probably caused by fading of the tracer in neurons after such a long period. It is not known exactly how long FB will stay in DRG neurons. Previous studies[23] have shown it to remain for at least 6 months in adult rat motoneurons. In the group with 12 months survival time, HRP seemed to be a better tracer (Table 1). The survival times were chosen since it can be expected that the cell loss is established after 6 months and that restitution of any significance should not be present after 12.[5,6] Neuronal counting always implies uncertainty of the exact number of cells. However, the correction methods used[26,27] diminish the variation due to counting errors and the statistical method using paired observations minimizes the interindividual variations. Therefore, the numbers estimated should be fully comparable between sides, although they might not be the actual numbers of cells.

## CONCLUSIONS

The present findings demonstrate no statistically significant difference between the number of surviving DRG cells after a peripheral nerve injury, whether it has been repaired using a PHB conduit or optimal direct suturing. In clinical practice, an optimal way of nerve repair is seldom applicable because of lacerated nerve ends. The need for revision results in suturing under tension or restoring continuity by grafting. An alternative to grafting is of great clinical interest. The use of a conduit at the site of injury has experimentally been demonstrated to improve peripheral regeneration.[15] Clinically, promising results of enclosing the nerve ends in silicone tubes have been found.[11] Enclosing the nerve ends in a conduit enhances the local concentration of growth factors like neurotrophins[13,28,41,42] and cellular elements such as macrophages.[36,43] Placing the lacerated ends in each end of a tube instead of trying to match them at the suture site could also leave the axons to find their way more accurately,[44] reducing the problem of iatrogenic mismatch.[45] A resorbable material like PHB also has the advantage of not requiring removal of the conduit to relieve a patient from local discomfort, which is needed when silicone is used.[11,34] Also, there will not be any risk of compression of the nerve by the material because of its elastic property. The present study demonstrates equal results for the resorbable conduit as for the optimal primary repair. The material seems to have a potential for being used as a conduit for nerve repair when the latter cannot be performed, even when there is a small nerve defect. It might also be possible to modify the regenerative capacity in severe cases by adding exogenous neurotrophic factors to the endogenic production in the tube, which may be an alternative to nerve grafts following extensive loss of nerve tissue.

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(54) Title: NEURAL REGENERATION CONDUIT

(57) Abstract: A neural regeneration conduit (10) employing spiral geometry (17) is disclosed. The spiral geometry is produced by rolling a flat sheet into a cylinder. The conduit can contain a multiplicity of functional layers lining the lumen of the conduit, including a confluent layer of adherent Schwann cells (126). The conduit can produce a neurotrophic agent concentration gradient by virtue of neurotrophic agent-laden microspheres (24) arranged in a nonuniform pattern and embedded in a polymer hydrogen layer lining the lumen of the conduit.

# NEURAL REGENERATION CONDUIT

## Cross-Reference to Related Applications

This application claims priority from U.S. Provisional Application Serial No. 60/179,201, filed January 31, 2000.

## Technical Field

This invention relates to neurology, cell biology and implantable prostheses, and particularly to methods and devices for surgical repair of transected or crushed nerves.

## Background of the Invention

Peripheral nerve defects have been repaired by means of surgically implanting autograft nerves and with various types of implanted prostheses. Hollow entubulation conduits, autologous materials, e.g., vein or muscle grafts, allograft nerves and combinations of these approaches have been attempted with limited success. Schwann cells in a nerve gap, delivery of neurotrophic agents and isolation of a local regenerating milieu have been implicated in peripheral nerve regeneration. However, practical devices and methods for efficiently combining these components are needed.

## Summary of the Invention

We have developed a neural regeneration conduit that employs spiral geometry. This advantageously permits formation of a multiplicity of functional layers lining the lumen of the conduit, including a confluent layer (e.g., monolayer) of adherent Schwann cells, and formation of neurotrophic agent concentration gradients.

The invention features a nerve regeneration conduit. The conduit includes: a porous biocompatible support which includes an inner surface and an outer surface, with the support being in the form of a roll. The roll is such that its cross section approximates a spiral spanning from 8 to 40 rotations, with the outer surface of the support facing outward, relative to the origin of the spiral. Preferably, a single layer of the support has a thickness of 5 μm to 200 μm, and more preferably 10 μm to 100 μm. The support can contain a naturally occurring biological material, for example, small intestinal submucosa (SIS), vein-derived tissue or acellular dermal

material. Alternatively, the support can contain a synthetic polymer. Suitable synthetic polymers include polyhydroxyalkanoates, e.g., polyhydroxybutyric acid; polyesters, e.g., polyglycolic acid (PGA); copolymers of glycolic acid and lactic acid (PLGA); copolymers of lactic acid and ε-aminocaproic acid; polycaprolactones; polydesoxazon (PDS); copolymers of hydroxybutyric acid and hydroxyvaleric acid; polyesters of succinic acid; polylactic acid (PLA); cross-linked hyaluronic acid; poly(organo)phosphazenes; biodegradable polyurethanes; and PGA cross-linked to collagen. In some embodiments, the support is bioresorbable.

Preferred embodiments of the invention include a layer of cells, for example, Schwann cells, adhered to the inner surface of the support. The conduit can contain from 15,000 to 165,000 Schwann cells per millimeter of conduit length. In some embodiments it contains from 20,000 to 40,000 Schwann cells per millimeter of conduit length, e.g., approximately 30,000 Schwann cells per millimeter of conduit length. The conduit can include a layer of extracellular matrix material, e.g., fibronectin, collagen or laminin, on the support.

The conduit can include a polymer hydrogel layer adhered to a layer of cells on the support, or to the support itself. Preferably the thickness of the hydrogel layer is 5 μm to 120 μm, and preferably 10 μm to 50 μm, e.g., approximately 25 μm. Materials suitable for use in a polymer hydrogel layer include fibrin glues, Pluronics®, polyethylene glycol (PEG) hydrogels, agarose gels, PolyHEMA (poly 2-hydroxyethylmethacrylate) hydrogels, PHPMA (poly N-(2-hydroxypropyl) methacrylamide) hydrogels, collagen gels, Matrigel®, chitosan gels, gel mixtures (e.g., of collagen, laminin, fibronectin), alginate gels, and collagen-glycosaminoglycan gels.

Some embodiments of the invention include a multiplicity of microspheres between the rolled layers of the support, e.g., immobilized in the hydrogel layer. The hydrogel layer can contain microspheres, a neurotrophic agent, or both. The neurotrophic agent can be incorporated directly into the hydrogel layer or loaded into microspheres. Suitable microsphere diameters range from of 1 μm to 150 μm. The microspheres can be formed from a material containing a copolymer of lactic acid and glycolic acid, preferably having an average molecular weight of 25 kD to 130 kD. In such a copolymer, the lactic acid:glycolic acid ratio can range from approximately 50:50 to almost 100% polylactic acid. In some embodiments, the ratio is approximately 85:15. Other materials also can be used to form the microspheres, e.g., polyhydroxyalkanoates, e.g., polyhydroxybutyric acid; polyesters, e.g., polyglycolic acid (PGA); copolymers of lactic acid and ε-aminocaproic acid; polycaprolactones; polydesoxazon (PDS);

copolymers of hydroxybutyric acid and hydroxyvaleric acid; polyesters of succinic acid; and cross-linked hyaluronic acid. The microspheres can be arranged in a pattern to facilitate creation of a neurotrophic agent concentration gradient. Such a gradient can be radial or axial. Examples of useful neurotrophic agents are FK506 (tacrolimus), αFGF (acidic fibroblast growth factor), βFGF (basic FGF), 4-methylcatechol, NGF (nerve growth factor), BDNF (brain derived neurotrophic factor), CNTF (ciliary neurotrophic factor), MNGF (motor nerve growth factor), NT-3 (neurotrophin-3), GDNF (glial cell line-derived neurotrophic factor), NT-4/5 (neurotrophin-4/5), CM101, inosine, spermine, spermidine, HSP-27 (heat shock protein-27), IGF-I (insulin-like growth factor), IGF-II (insulin-like growth factor 2), PDGF (platelet derived growth factor) including PDGF-BB and PDGF-AB, ARIA (acetylcholine receptor inducing activity), LIF (leukemia inhibitory factor), VIP (vasoactive intestinal peptide), GGF (glial growth factor), IL-1 (interleukin-1), and neurotrophic pyrimidine derivative MS-430. The hydrogel layer can contain two or more neurotrophic agents. Different neurotrophic agents can be loaded into separate batches of microspheres, or two or more neurotrophic agents can be loaded into a single batch of microspheres.

The invention also features a method of manufacturing a nerve regeneration conduit. The method includes providing a porous, biocompatible support having an inner surface and an outer surface; and forming the support into a roll such that a cross section of the roll approximates a spiral spanning from 8 to 40 rotations, with the outer surface of the support facing outward, relative to the origin of the spiral. In addition, the method can include one or more of the following: culturing a layer (e.g., a monolayer) of cells on the support before forming the support into the roll, depositing a hydrogel layer and/or a multiplicity of microspheres on the support before forming the support into a role, loading a neurotrophic agent into the microspheres, and arranging the microspheres in a nonuniform pattern to facilitate neurotrophic agent concentration gradient formation.

The invention also features a method of facilitating regeneration of a transected nerve across a nerve gap defined by a proximal end of the transected nerve and a distal end of the transected nerve. The method includes: coapting the proximal end of the transected nerve to a first end of the conduit, and coapting the distal end of the transected nerve to a second end of the conduit.

The invention also features a method of facilitating regeneration of a crushed nerve. The method includes: providing a porous biocompatible support having an inner surface and an outer surface; culturing a layer of neurological cells (e.g., Schwann cells) on the support; and rolling the support around the crushed nerve. The method also can include depositing a hydrogel layer on the support before rolling the support around the crushed nerve, or incorporating a neurotrophic agent (e.g., via a microsphere or directly) into the hydrogel.

As used herein, "neurotrophic agent" means neurotropin or neurotrophin, i.e., any molecule that promotes or directs the growth of (1) neurons or portions thereof (e.g., axons), or (2) nerve support cells such as glial cells (e.g., Schwann cells).

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims. All publications and other documents cited herein are incorporated by reference.

## Brief Description of the Drawings

FIG. 1A is a schematic cross sectional view of a partially-rolled nerve regeneration conduit.

FIG. 1B is a schematic cross sectional view of a portion of a multilayered sheet used to form the nerve regeneration conduit in FIG 1A.

FIG 2A is a schematic top view onto the inside surface of an unrolled conduit of the invention.

FIG 2B is a cross-sectional view of the unrolled conduit shown in FIG 2A, taken at line A-A.

FIG 2C is an end view of the conduit shown in FIGS 2A and 2B, partially rolled according to arrow B in FIGS 2A and 2B.

Like reference symbols in the various drawings indicate like elements.

## Detailed Description of the Invention

The invention exploits the considerable advantages of "rolled architecture" in neural regeneration conduit. In rolled architecture, axial channels are replaced by a single spiraling axial space. This provides several advantages, including one or more of the following: (1)

increased surface area for adherence of neural regeneration-supporting cells inside the conduit and to guide regeneration of an injured nerve; (2) a polymer hydrogel layer that provides an aqueous milieu for cell migration and neurotrophic agent diffusion; and (3) neurotrophic agents loaded into microspheres lining the inside of the conduit; (4) non-uniform geographic arrangement of microspheres to create axial or radial concentration gradient(s) of a single neurotrophic agent or multiple neurotrophic agents; (5) creation of a spatial gap (to accommodate regenerating nerves) by a hydrogel/microsphere layer acting as a spacer, or spacers joined or contiguous with the support, along the inside of the conduit; (6) choice of conduit materials; and (7) ease of manufacturing.

FIG. 1A is a cross sectional view of a partially-rolled nerve regeneration conduit 10. A porous support 12 has an outer surface 13 and an inner surface 15. An approximately spiral lumen 14 is created by rolling the support 12. Formation of a uniform space 14 between rolled layers of the support 12 is facilitated by a semi rigid hydrogel/microsphere layer (shown in FIG 1B) adhered to the inner surface 15 of the support. The outer surface 13 faces outward with respect to the origin 16 of the spiral 17, and the inner surface 15 faces inward with respect to the origin 16 of the spiral 17. For ease of depiction, the schematic representation shows a partially-rolled conduit, whose spiral 17 lumen contains only approximately 3½ rotations. In preferred embodiments of the invention the spiral 17 contains from 8 to 40 rotations. The number of rotations will depend on various factors, including thickness of the support, thickness of the gap between support layers, and the desired outside diameter of the fully-rolled, cylindrical conduit. The conduit can be designed to have an outside diameter approximately matching the diameter of the nerve in which a gap is being bridged.

FIG. 1B is a schematic, cross sectional view of a portion of a multilayered sheet 20 used to form the nerve regeneration conduit 10. A layer of Schwann cells 26 is adhered to the inner surface 15 of the porous support 12. Neurotrophin-laden microspheres 24 are embedded in a hydrogel layer 22.

Referring to FIGS 2A-2C, an alternative embodiment of a conduit is shown. FIG 2A is a top view of an unrolled sheet 120, showing inside surface 115. Instead of a hydrogel layer providing spacing between layers of a roll, sheet 120 includes continuous spacers 130 and discontinuous spacers 132 (FIG 2C). Of course, in other embodiments, a sheet can include either continuous or discontinuous spacers only. These spacers 130 and 132 and the rest of the sheet

120 can be formed from any castable foam material that is suitable for implantation, produced using microfabrication techniques, or formed using ink jet technology as described herein. Schwann cells 126 are adhered on inside surface 115. To form a rolled conduit 110, sheet 120 is rolled in direction B shown in FIGS 2A and 2B. Rolled conduit 110 has outside surface 113.

Conduit 110 also includes an axial gradient of neurotrophin molecules 134 which are loaded into spacers 130 and 132. Such a gradient can be provided when the spacers and/or sheet is fabricated by ink jet technology. Alternatively, conduit 110 can be used in conjunction with microspheres and/or a hydrogel (not shown) that contain one or more neurotrophins, the microspheres being positioned between spacers 130 and 132.

Conduit support

There is considerable latitude in material used to form the conduit support 12. The material must be porous and biocompatible. In addition, it must have suppleness or ductility sufficient to permit rolling of the support into a compact, cylindrical structure, e.g., having a diameter approximately 0.5 to 3.0 mm, suitable for surgical implantation in the repair of transected or crushed nerves. Preferably, the support can be cut readily with surgical instruments, yet strong enough to anchor surgical sutures. In embodiments incorporating a layer of cells, the support should allow for adherence of cells. It is, however, important to note that cell adherence is not necessary for the operation of the invention. The thickness of the support 12 (single layer) can vary. Preferably it is from 5 to 200 μm, and more preferably, it is from 10 to 150 μm. Optimal thickness will depend on the material used to form the support 12, the size and anatomical location of the nerve to be repaired, and the length of the nerve gap (if any) to be bridged in the repair. After being formed by rolling, the cylindrical nerve conduit preferably displays at least some flexibility.

In some embodiments of the invention, the support 12 is formed partly or completely from a naturally occurring biological material. A suitable naturally occurring biological material is small intestinal submucosa (SIS). SIS is an acellular collagen matrix that contains endogenous growth factors and other extracellular matrix components. Techniques for harvesting and handling SIS are known in the art. See, e.g., Lantz et al., J. Invest. Surg. 6:297-310 (1993). Other potentially useful natural, biological materials are vein tissue and acellular material. In many embodiments of the invention, the support contains only non-immunogenic components.

For example, SIS in not immunogenic. If immunogenic components are used, suitable immunosuppressive therapy may be necessary. Such immunotherapy is known to those of skill in the art. See, e.g., Evans et al., Progress in Neurobiology 43:187-233, 1994.

In some embodiments of the invention, the support 12 is a thin sheet of synthetic polymer. Suitable synthetic polymers include polyhydroxyalkanoates, e.g., polyhydroxybutyric acid; polyesters, e.g., polyglycolic acid (PGA); copolymers of glycolic acid and lactic acid (PLGA); copolymers of lactic acid and ε-aminocaproic acid; polycaprolactones; polydesoxazon (PDS); copolymers of hydroxybutyric acid and hydroxyvaleric acid; polyesters of succinic acid; polylactic acid (PLA); cross-linked hyaluronic acid; poly(organo)phosphazenes; biodegradable polyurethanes; and PGA cross-linked to collagen. Poly(organo)phosphazene supports are described in Langone et al., Biomaterials 16:347-353, 1995. Polyurethane supports are described in Robinson et al., Microsurgery 12:412-419, 1991. The support can be bioresorbable, e.g., PLGA, or nonbioresorbable, e.g., SIS. In addition, the inclusion of an electrically conducting polymer (e.g., oxidized polypyrrole) in the conduit, in conjunction with electrical stimulation, can augment nerve repair. Such a strategy is described in Schmidt et al., Proc. Natl. Acad. Sci. USA 94:8948-8953, 1997.

The support and any structures contiguous with it (e.g., spacers) can be fabricated using any method known in the art. For example, the use of foam casting for generating prosthetic sheets with varying porosity can be adapted from processes described in Nam et al., Biomaterials 20:1783-1790, 1999; Nam et al., J. Biomed. Mat. Res. 47:8-17, 1999; and Schugens et al., J. Biomed. Mat. Res. 30:449-461, 1996. The porosity of biomaterials formed from casting can be controlled using differential concentrations of salts or sugars, $CO_2$ gas pressure, and other means known in the art. See, e.g., Lu et al., Biomaterials 21:1595-1605, 2000; Harris et al., J. Biomed. Mat. Res. 42:396-402, 1998; and Wake et al., Cell Transplantation 5:465-473, 1996. The pores in the foam should be large enough for exchange of gases and nutrients as necessary for cell maintenance, but small enough so that the surface of the support is impermeable to cells. A typical range suitable for a support of the invention is about 10-100 μm.

As an alternative to foam casting, microfabrication is a process that includes casting a polymer on top of a silicon wafer that has been etched. Most common polymers used in this process include polydimethylsiloxane (PDMS), which is non-biodegradable. However,

microfabrication techniques can be adapted for biodegradable PLGA and the like, using a modification of the procedure described in Becker, Electrophoresis 21:12-26, 2000.

In some embodiments of the invention, it is desirable to deposit or impregnate the support with neurotrophins (e.g., a gradient of one or more neurotrophins) for facilitating axon migration and nerve regeneration in general. One means of accomplishing this task is to incorporate three-dimensional printing (3DP) ink jet printing technology into the manufacture of the support to produce a gradient of neurotrophins. General 3DP techniques as applied to medical devices is described in U.S. Patent Nos. 5,490,962 and 5,869,170. If a gradient is not desired, a number of art-recognized methods can be used evenly distribute neurotropins throughout a support.

Layer of cells

In some embodiments of the invention, a monolayer of adherent cells 26 is cultured on the support 12 before it is rolled into a cylinder. Preferably, the cells 26 remain adhered to the support after the support is rolled into a cylinder for implantation. The cells 26 are employed for their ability to promote axonal extension of neurons in nerves. Schwann cells are particularly suitable, but any other adherent cell that promotes axonal extension can be employed. Alternatively, even if the Schwann cells do not adhere to the support, the cells can be encapsulated in the hydrogel described herein. Schwann cells encapsulated in hydrogels are described in Plant et al., Cell Transplantation 7:381-391, 1998; and Guenard et al., J. Neurosci. 12:3310-3320, 1992.

It is envisioned that a variety of cells can be included in the conduit to facilitate nerve regeneration. For example, the harvesting and use of olfactory ensheathing glial cells in nerve regeneration is described in Verdu et al., Neuroreport 10:1097-1101, 1999; and Ramon-Cueto et al., J. Neurosci. 18:3803-3815, 1998. In addition, neural stem cells, neural crest stem cells, or neuroepithelial cells can be harvested and optionally differentiated into neural support cells, such as described in Mujtaba et al., Dev. Biol. 200:1-15, 2000; Pardo et al., J. Neurosci. Res. 59:504-512, 2000; Mytilineou et al., Neurosci. Lett. 135:62-66, 1992; and Murphy et al., J. Neurosci. Res. 25:463-475, 1990. Alternatively, autologous bone marrow stromal cells can be differentiated into neural stem cells for use in a conduit. This conduit can then be grafted into the donor for nerve repair without the concern for graft rejection arising from implantation of allogenic or xenogenic cells. Isolation and differentiation of bone marrow stromal cells are

described in Woodbury et al., J. Neurosci. Res. 61:364-370, 2000; and Sanchez-Ramos et al., Exp. Neurol. 164:247-256, 2000.

Optionally, the cells employed in the monolayer 26 are genetically engineered for one or more desirable traits, e.g., overexpression of a neurotrophic factor or axonal extension-promoting protein. Such cells need not be of glial cell origin, since the recombinant expression of neurotrophic factor in non-glial cells renders them suitable for use in the invention. In other words, recombinant expression converts originally non-nerve support cells into nerve support cells. Fibroblasts that express neurotrophins and are suitable for implantation are described in Nakahara et al., Cell Transplantation 5:191-204, 1996. Examples of axonal extension-promoting proteins include NGF (Kaechi et al., J. Pharm. Exp. Ther. 272:1300-1304, 1995), FGF (Laird et al., Neuroscience 65:209-216, 1995), and GDNF (Frostic et al., Microsurgery 18:397-405, 1998). Other neurotrophins include FK506, 4-methylcatechol, BDNF, CNTF, MNGF, NT-3, NT-4/5, CM101, inosine, spermine, spermidine, HSP-27, IGF-I, IGF-II, PDGF (including PDGF-BB and PDGF-AB), IL-1, ARIA, LIF, VIP, GGF, and MS-430.

Production of a confluent layer of cells 26 on the support 12 can be accomplished readily through cell culture, using a mitogenic medium, and conventional animal cell culture techniques and equipment. Conventional cell culture techniques are known in the art and can found in standard references. See, e.g., Casella et al., Glia 17:327-338 (1996); Morrissey et al., J. Neuroscience 11:2433-2442 (1991).

In other embodiments, the cells can be grown on both the inside and outside surfaces of a support.

Hydrogel layer

Some embodiments of the invention include a polymer hydrogel layer 22 adhered to the support 12 or to a layer of cells 26 adhered to the support 12. The polymer hydrogel layer 22 can be any biocompatible, bioresorbable polymer gel that provides an aqueous milieu for cell migration and neurotrophic agent diffusion. The hydrogel can be natural or synthetic. The hydrogel layer 22 can have a thickness from 5 to 120 μm, preferably from 10 to 50 μm, e.g., approximately 20, 25 or 30 μm. Optimal hydrogel thickness depends on factors such as the diameter of the nerve being repaired and the number and diameter of microspheres 24 (if any) to be accommodated in the hydrogel layer 22. Exemplary materials for use in a polymer hydrogel

layer 22 are fibrin glues, Pluronics®, polyethylene glycol (PEG) hydrogels, agarose gels, PolyHEMA (poly 2-hydroxyethylmethacrylate) hydrogels, PHPMA (poly N-(2-hydroxypropyl) methacrylamide) hydrogels, collagen gels, Matrigel®, chitosan gels, gel mixtures (e.g., of collagen, laminin, fibronectin), alginate gels, and collagen-glycosaminoglycan gels. The hydrogel layer 22 can contain one or more neurotrophic agents or axon extension-promoting proteins. Such neurotrophic agents can be loaded directly into the hydrogel 22, loaded into microspheres 24, or incorporated into the support or spacers as described herein.

Microspheres

Some embodiments of the invention include microspheres between the rolled layers of the support. The microspheres can be held in place by any suitable means. For example, the microspheres can be immobilized in the hydrogel layer. The microspheres can be "blank," i.e., containing no active ingredient. Blank microspheres are can serve as spacers to aid in producing a desired and constant spacing between laminations of the support in the spiral. Microspheres 24 useful in the invention can have diameters of approximately 1 μm to 150 μm. Preferably, the microspheres are made of a semi rigid, biocompatible, bioresorbable polymeric material. A suitable polymeric material is a high molecular weight (approx. 130 kD) copolymer of lactic acid and glycolic acid (PLGA). PLGA is well tolerated in vivo, and its degradation time can be adjusted by altering the ratio of the two co-monomers.

Besides serving as spacers, microspheres can be loaded with one or more neurotrophic agents, or any other active ingredient, so that they serve as drug delivery vehicles. Effective use of PLGA as a drug delivery vehicle is known in the art. See, e.g., Langer, Ann. of Biomed. Eng. 23:101, 1995; and Lewis, "Controlled release of bioactive agents from lactide/glycolide polymers," in Chasin and Langer (eds.), Biodegradable Polymers as Drug Delivery Systems, Marcel Dekker, New York (1995).

A particularly advantageous feature of the invention is that microspheres loaded with a neurotrophic agent can be arranged in a pattern so as to result in an axial or radial concentration gradient in the lumen of the nerve regeneration conduit. Moreover, when two or more neurotrophic agents are employed, the agents can be loaded into separate batches of microspheres, which can then be differently arranged to produce independent concentration gradients for each of the different neurotrophic agents. Effects of neurotrophin concentration

gradients are known in the art. See, e.g., Goodman et al., Cell 72:77-98, 1993; and Zheng et al., J. Neurobiol. 42:212-219, 2000. Utilization of such concentration gradient effects is within ordinary skill in the art. In some embodiments of the invention designed to create a neurotrophic agent concentration gradient, the two ends of the conduit differ from each other with respect to one or more neurotrophic agents. Such conduits may require implantation across a nerve gap in only one of two possible orientations. To ensure implantation in the proper orientation, the two ends of the conduit can be rendered visually distinguishable by any suitable means, e.g., a non-toxic dye marking on the conduit itself, or markings on a sterile wrapper or container.

Surgical procedures

Surgical procedures known in the art can be employed when using a nerve regeneration conduit of the invention to repair transected peripheral nerves. Suitable surgical procedures are described, for example, in Hadlock et al., Archives of Otolaryngology – Head & Neck Surgery 124:1081-1086, 1998; WO 99/11181; U.S. Patent No. 5,925,053; WO 88/06871; Wang et al., Microsurgery 14:608-618, 1993; and Mackinnon et al., Plast. Reconst. Surg. 85:419-424, 1990.

Example

Schwann cells were isolated from neonatal Fisher rats. Small intestinal submucosa (SIS) was harvested from adult Fisher rats for use as a support material in a nerve regeneration conduit. The SIS was cut into 7 mm by 8 cm pieces and pinned out. Schwann cells were plated onto the SIS sheets and cultured until they reached confluence. The strips were then rolled into a laminar structure and implanted across a 7 mm gap in the rat sciatic nerve (n = 12). Control animals received SIS conduits without Schwann cells (n = 11) or an autograft repair (n = 12).

At both 6 and 10½ weeks, functional recovery through the Schwann cell-laden SIS conduits, measured by sciatic function index, exceeded that through the cell-free conduits, but compared favorably with autografts.

Other embodiments

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

What is claimed is:

1. A nerve regeneration conduit comprising a porous biocompatible support comprising an inner surface and an outer surface, the support being in the form of a roll such that a cross section of the roll approximates a spiral spanning from 8 to 40 rotations, with the outer surface of the support facing outward, relative to the origin of the spiral.

2. The nerve regeneration conduit of claim 1, wherein the support has a thickness of 5 to 200 μm.

3. The nerve regeneration conduit of claim 1, wherein the support has a thickness of 10 to 100 μm.

4. The nerve regeneration conduit of claim 1, wherein the support comprises a biological material.

5. The nerve regeneration conduit of claim 4, wherein the biological material is small intestinal submucosa.

6. The nerve regeneration conduit of claim 1, wherein the support comprises a synthetic polymer.

7. The nerve regeneration conduit of claim 1, wherein the support is bioresorbable.

8. The nerve regeneration conduit of claim 6, wherein the synthetic polymer is selected from the group consisting of polyhydroxyalkanoates, e.g., polyhydroxybutyric acid; polyesters, e.g., polyglycolic acid (PGA); copolymers of glycolic acid and lactic acid (PLGA); copolymers of lactic acid and ε-aminocaproic acid; polycaprolactones; polydesoxazon (PDS); copolymers of hydroxybutyric acid and hydroxyvaleric acid; polyesters of succinic acid; polylactic acid (PLA); cross-linked hyaluronic acid; poly(organo)phosphazenes; biodegradable polyurethanes; and PGA cross-linked to collagen.

9. The nerve regeneration conduit of claim 1, further comprising a layer of cells adhered to the inner surface of the support.

10. The nerve regeneration conduit of claim 9, wherein the cells are Schwann cells or olfactory ensheathing glial cells.

11. The nerve regeneration conduit of claim 10, wherein the layer contains from 15,000 to 165,000 Schwann cells per millimeter of conduit length.

12. The nerve regeneration conduit of claim 11, wherein the layer contains from 20,000 to 40,000 Schwann cells per millimeter of conduit length.

13. The nerve regeneration conduit of claim 9, further comprising a layer of extracellular matrix material on the support.

14. The nerve regeneration conduit of claim 1, further comprising a hydrogel layer.

15. The nerve regeneration conduit of claim 14, wherein the hydrogel layer has a thickness of 5 to 120 μm.

16. The nerve regeneration conduit of claim 15, wherein the hydrogel layer has a thickness of 10 to 50 μm.

17. The nerve regeneration conduit of claim 14, wherein the hydrogel layer comprises a polymer selected from the group consisting of fibrin glues, Pluronics®, polyethylene glycol (PEG) hydrogels, agarose gels, PolyHEMA (poly 2-hydroxyethylmethacrylate) hydrogels, PHPMA (poly N-(2-hydroxypropyl) methacrylamide) hydrogels, collagen gels, Matrigel®, chitosan gels, gel mixtures (e.g., of collagen, laminin, fibronectin), alginate gels, and collagen-glycosaminoglycan gels.

18. The nerve regeneration conduit of claim 1, further comprising a multiplicity of microspheres.

19. The nerve regeneration conduit of claim 18, wherein the microspheres are immobilized in a hydrogel layer.

20. The nerve regeneration conduit of claim 14, wherein the hydrogel layer comprises a neurotrophic agent.

21. The nerve regeneration conduit of claim 18, wherein the microspheres comprise a neurotrophic agent.

22. The nerve regeneration conduit of claim 18, wherein the microspheres have a diameter of 1 to 150 μm.

23. The nerve regeneration conduit of claim 18, wherein the microspheres comprise a material selected from the group consisting of a polyhydroxyalkanoate, a polyester, a copolymer of glycolic acid and lactic acid (PLGA), a copolymer of lactic acid and ε-aminocaproic acid, a polycaprolactones, polydesoxazon (PDS), a copolymer of hydroxybutyric acid and hydroxyvaleric acid, a polyester of succinic acid; and cross-linked hyaluronic acid.

24. The nerve regeneration conduit of claim 23, wherein the microspheres comprise PLGA having an average molecular weight of 25 kD to130 kD.

25. The nerve regeneration conduit of claim 24, wherein the lactic acid:glycolic acid ratio is approximately 85:15.

26. The nerve regeneration conduit of claim 18, wherein the microspheres are arranged in a pattern to facilitate creation of a neurotrophic agent concentration gradient.

27. The nerve regeneration conduit of claim 26, wherein the gradient is radial.

28. The nerve regeneration conduit of claim 26, wherein the gradient is axial.

29. The nerve regeneration conduit of claim 20 or 21, wherein the neurotrophic agent is selected from the group consisting of FK506, αFGF, βFGF, 4-methylcatechol, NGF, BDNF, CNTF, MNGF, NT-3, GDNF, NT-4/5, CM101, inosine, spermine, spermidine, HSP-27, IGF-I, IGF-II, PDGF, ARIA, LIF, VIP, GGF, IL-1, and MS-430.

30. The nerve regeneration conduit of claim 20, wherein the hydrogel layer comprises two or more neurotrophic agents.

31. The nerve regeneration conduit of claim 21, wherein the microspheres comprise two or more neurotrophic agents.

32. The nerve regeneration conduit of claim 31, wherein the neurotrophic agents are in separate microspheres.

33. The nerve regeneration conduit of claim 31, wherein two or more neurotrophic agents are in a single microsphere.

34. A method of manufacturing a nerve regeneration conduit, the method comprising providing a porous biocompatible support comprising an inner surface and an outer surface; and forming the support into a roll such that a cross section of the roll approximates a spiral spanning from 8 to 40 rotations, with the outer surface of the support facing outward, relative to the origin of the spiral.

35. The method of claim 34, further comprising culturing a layer of cells on the support prior to forming the support into the roll.

36. The method of claim 34, further comprising depositing a hydrogel layer on the support before forming the support into a roll.

37. The method of claim 34, further comprising incorporating a multiplicity of microspheres into the conduit.

38. The method of claim 37, wherein the microspheres comprise a neurotrophic agent.

39. A method of facilitating regeneration of a transected nerve across a nerve gap defined by a proximal end of the transected nerve and a distal end of the transected nerve, the method comprising coapting the proximal end of the transected nerve to a first end of the conduit of claim 1, and coapting the distal end of the transected nerve to a second end of the conduit.

40. A method of facilitating regeneration of a crushed nerve, the method comprising providing a porous biocompatible support comprising an inner surface and an outer surface; culturing a layer of cells on the support; and rolling the support around the crushed nerve.

41. The method of claim 40, further comprising depositing a hydrogel layer on the support before rolling the support around the crushed nerve.

42. The method of claim 40, further comprising incorporating a multiplicity of neurotrophic agent-laden microspheres into the conduit.

43. The nerve regenerating conduit of claim 14, wherein the hydrogel further comprises cells.

44. The nerve regenerating conduit of claim 1, wherein the support further comprises spacer members extending from the inner surface of the support.

45. The nerve regenerating conduit of claim 1, wherein the support is loaded with one or more neurotrophins.

46. The nerve regenerating conduit of claim 45, wherein the one or more neurotrophins are distributed in a gradient in the support.

FIG. 1A

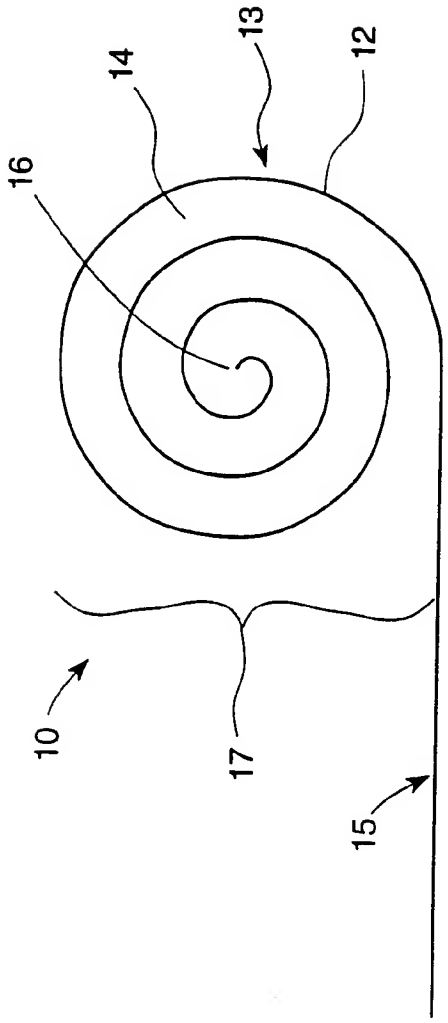
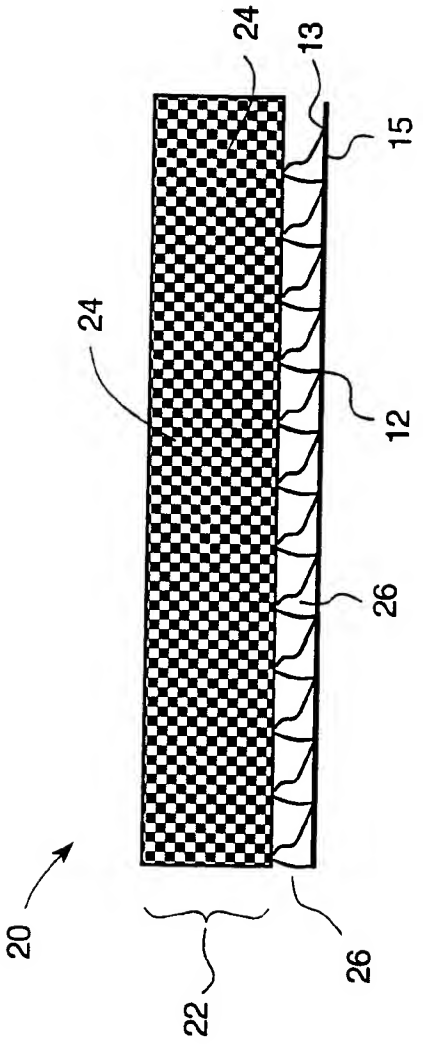



FIG. 1B



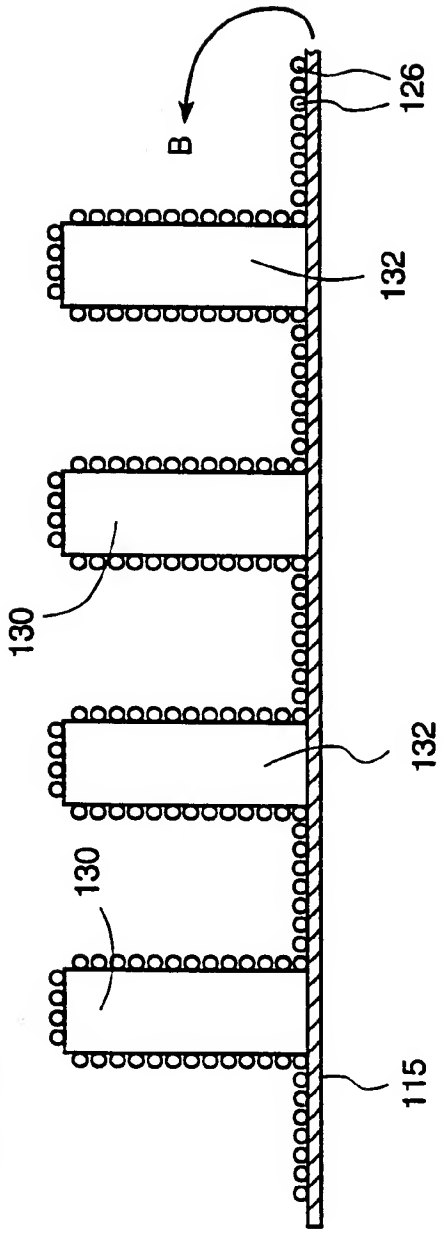



FIG. 2B

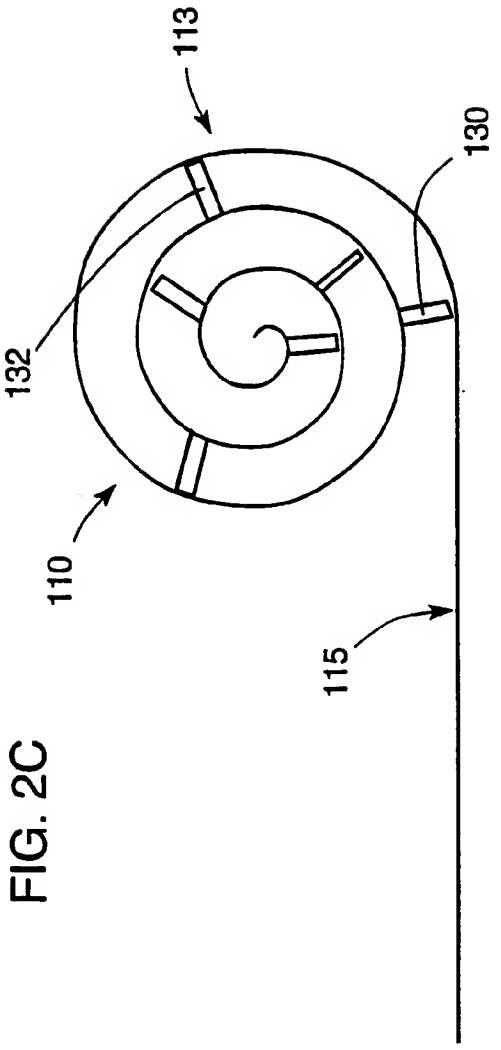



FIG. 2C

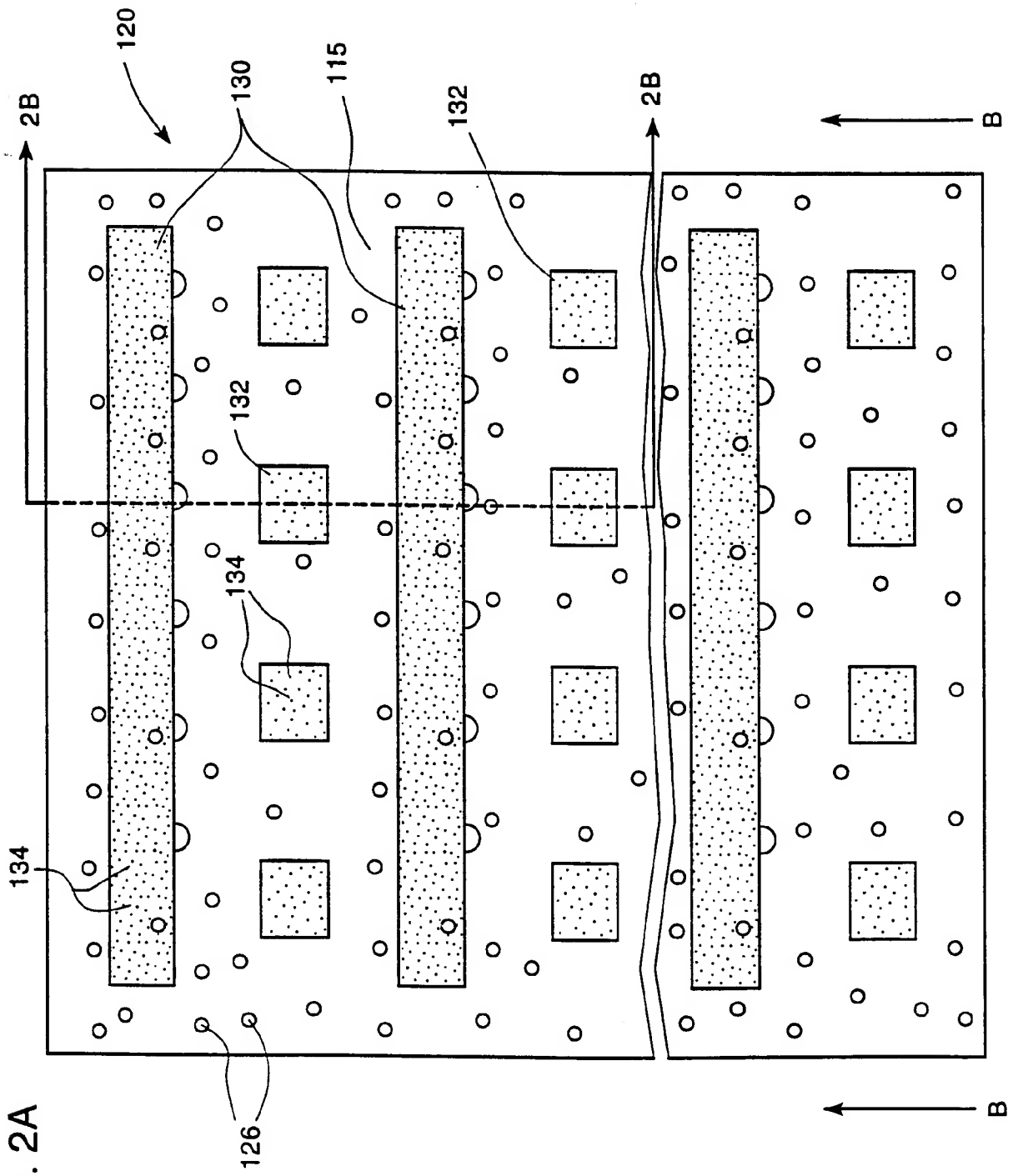



FIG. 2A

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/03122

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61B 17/08
US CL : 606/152

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 606/152

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

E.A.S.T.

search terms: nerve regeneration, conduit, spiral, roll

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | US 4,778,467 A (STENSAAS et al) 18 October 1988, col. 2, lines 13-68 and col. 3, lines 1-2. | 1-33, 43-46NO |
| A | US 5,122,151 A (de Medinaceli) 16 June 1992, col. 4, lines 14-53. | 1-33, 43-46 |
| A | US 5,948,020 A (YOON et al) 07 September 1999, see Abstract of the Disclosure. | 1-33, 43-46 |
| A | US 5,400,784 A (DURAND et al) 28 March 1995, see abstract of the Disclosure. | 1-33, 43-46 |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
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| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 23 MAY 2001 | 06 JUL 2001 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230 | Authorized officer<br>GARY JACKSON<br>Telephone No. (703) 308-4302 |

Form PCT/ISA/210 (second sheet) (July 1998) ★

# INTERNATIONAL SEARCH REPORT

Internati application No.
PCT/US01/03122

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 34-42
because they relate to subject matter not required to be searched by this Authority, namely:

The above claims are directed to non-statutory matter of a method of performing a surgical procedure. PCT Article 17(2)(a); Rule 39.1(iv).

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

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(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**
*with international search report*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 03/041758 A1

(54) Title: NERVE REPAIR UNIT AND METHOD OF PRODUCING IT

(57) Abstract: A nerve repair unit comprising a resorbable polymeric support and an alginate matrix containing human Schwann cells is enclosed. A method of producing the nerve repair unit is also described. The Schwann cells are preferably cells cultured from a nerve biopsy sample from the patient who is going to receive the nerve repair unit as an implant.

# Nerve repair unit and method of producing it

The present invention relates to a nerve repair unit and a method of producing it. The nerve repair unit comprises a resorbable polymeric support and an alginate matrix containing human Schwann cells.

## BACKGROUND

It is well known that neural cells undergo changes after nerve injury, and some cells die. Further, it is known today that this cell death is extensive and results in changes of the projection pattern that the injured nerve has on the spinal cord level. Despite optimal microsurgical repair immediately after an induced experimental animal nerve injury a 25 –50 % loss of nerve cells arises, and is accompanied by an even greater loss of sensory reflex contacts inside the spinal cord (1-8). This nerve cell death is initiated within the first few days after the nerve injury and continues for several months (9). This cell death contributes to the impaired recovery of sensation in patients with nerve injuries, since a loss of nerve cells makes it more difficult for outgrowth of sensory nerves to the target area (10).

For reduction of this cell death, groups of small proteins (growth factors) are used, which are of great importance for the nerve cell function and survival. At nerve injury, these factors are released from cells, so-called Swann cells, that surround the injured nerve ends. However, due to leakage to the surrounding tissue, the concentration does not become high enough to prevent the nerve cell death. By introducing these factors into the spinal cord canal during the nerve healing, it has been demonstrated on rat that they can completely eliminate the nerve cell death (11).

In clinical practice most patients with major proximal nerve injuries (so-called plexus injuries) after difficult childbirth and traffic accidents are subjected to surgery after 4-12 weeks, since this time is necessary for evaluating the extent of the nerve injuries.

It would be desirable, especially in these emergency cases, when the patients come to the hospital, to take a microscopic nerve biopsy for cultivation of the patient's own Schwann cells. At the time for reconstruction of the injured area, it would be desirable to have means for substituting lost nerve tissue with the patient's own Schwann cells.

## DESCRIPTION OF THE INVENTION

The present invention provides means for repair of injured human nerves. Thus, the invention is directed to a nerve repair unit comprising a resorbable polymeric support and an alginate matrix containing human Schwann cells.

The resorbable polymeric support may be any commercially available support or support described in the literature suitable for implantation, especially a resorbable conduit for nerve regeneration.

In an embodiment of the invention the polymer of the resorbable polymeric support is selected from the group consisting of polyhydroxybutyric acid, polyglycolic acid and polylactic acid. In a presently preferred embodiment the polymer is polyhydroxybutyric acid (PHB).

In a presently most preferred embodiment the resorbable polymeric support is a polyhydroxybutyric acid conduit.

The choice of alginate as the matrix to be used in the present invention was preceded by experimental work evaluation also Collagen Type I (Sigma: C7661); Fibrin glue (Tisseel™, Immuno); Hyaluronic acid (Hylan G-F 20, Biomatrix) and Matrigel®: growth factor reduced Matrigel (Collaborative Biomedical Products, Becton Dickinson Labware). They were all inferior to alginate for the purpose of the present invention.

In an embodiment of the invention the alginate matrix is an ultrapure, low viscosity mannuronic acid alginate.

The human Schwann cells may be received from a culture of a biopsy sample from e.g. a branch of the sural nerve of the leg of a donor. Preferably the donor is the patient who is to receive the nerve repair unit of the invention. In case the patient has an open injury, a nerve biopsy sample may be taken from the damaged peripheral nerve for culturing of the Schwann cells.

Another aspect of the invention is directed to a method of producing a nerve repair unit comprising a resorbable polymeric support and an alginate matrix containing human Schwann cells. The method comprises the steps of mixing an alginate in isotonic saline solution with a human Schwann cell suspension in fibronectin, supplying the mixture to the resorbable polymeric support, setting the alginate matrix with a solution of calcium chloride in human cell culture medium, rinsing with the human cell culture medium and keeping the nerve repair unit in the human cell culture medium until use.

In an embodiment of the method of the invention the polymer of the resorbable polymeric support is selected from the group consisting of polyhydroxybutyric acid, polyglycolic acid and polylactic acid, the alginate is ultrapure, low viscosity mannuronic acid alginate and the human cell culture medium is Dublbecco's Minimum Eagles Medium plus Glutamax® (DMEM).

In another embodiment of the method of the invention the human Schwann cells are produced by transporting a sample of a peripheral nerve of a human patient in a transfer medium at ambient temperature within 24 hours to a competent laboratory, upon arrival washing the sample with a human culture medium, removing the perineurum, dividing the nerve branches into fascicles and cutting them into pieces, washing the pieces with a human culture medium and placing them into a culture flask coated with both poly-D-lysine and human laminin and covering them with a Schwann cell culture medium, followed by incubation at 37°C for up to 10 days, changing the medium every 2 days, and removing the nerve segments for digestion in a container with cell culture medium plus collagenase I and dispase I until the segments have broken down, followed by trituration, filtration, washing and centrifugation, resuspending the resulting pellet in transfer medium and plating the suspension on a culture flask coated with both poly-D-lysine and human laminin and after 24 hours changing the medium to a Schwann cell culture medium, followed by cultivation for at least 7 days under conditions removing possible fibroblast contamination and changes of culture medium until the Schwann cells are confluent and ready to split, aspirating the medium and suspending the cells in a trypsinized solvent, followed by centrifugation and washing of the pellet, counting the Schwann cells with a haematocytometer and plating them on a culture flask coated with both poly-D-lysine and human laminin and covering them with a Schwann cell culture medium at a density of 5x $10^5$ cells/ 25 $cm^2$, culturing the cells for at least 14 days under change of medium every day to produce an appropriate cell number for transplantation to the patient.

In the experimental part of this description it is shown, in an animal model, that allogenic cells have a survival time that is shorter compared to syngenic cells *in vivo* when no immunosuppression is used. However, allogenic Schwann cells have a survival time, prior to rejection, that may be sufficient for minor nerve injuries with shorter defects in the nerve tissue.

The invention will now be illustrated by description of experiments, but it should be understood that the scope of protection is not limited to specifically mentioned details.

## EXPERIMENTS

### Materials used in the experiments

**Resorbable polymer**

| | |
|---|---|
| Poly hydroxy butyrate (PHB) sheets | Astra-tech, Sweden |

**Matrix**

| | |
|---|---|
| Alginate, low viscosity mannuronate (LVM) Batch no: 210-241-02 | Pronova Biomedical, Norway |
| Calcium chloride | Sigma |
| Fibronectin, 0.1%, bovine plasma | Sigma |

**Tissue culture**

| | |
|---|---|
| Cell growth medium | DMEM plus: 10% fetal calf serum and Penicillin/Streptomycin (100iu-100µg) |
| Collagenase I | Worthington Biochemicals, Code; CLS-I, 125 U/ mg. 1% working solution made with DMEM and aliquoted in 300 µl and kept at -20°C. |
| Complement, rabbit anti mouse | Cedarlane Labs, Cat No: CL3051, 2 vials reconstituted at a time, kept on ice at all times, 1ml sterile water added to each vial, filtered through 0.45µM Filter, stored in liquid Nitrogen as 250 µl aliquots. |
| Cytosine-β-D-arabinofuranoside 5'-tri-Phosphate (Arac-C) | Sigma, Cat No: C3639/C8779, Stock solution ~1mM: 2 mg dissolved in 4 ml PBS, filtered 0.2 µm and stored at –20 °C, 250 µl aliquots |
| Dispase I: | Roche, Cat No: 210 455, 5 mg. Stock solution: 1 ml of sterile water added, stored at –20 °C as 0.1 ml aliquots, i.e. each aliquot ~ 3 U. |
| DMEM with HEPES | Gibco, Cat No: 22320 |
| DMEM, high glucose (4.5 g/l glucose) | Gibco, Cat No |
| Dulbecco's Modified Eagle's Medium plus Glutamax (DMEM) | Gibco, Cat No: 21885 |
| Fetal Calf Serum (FCS) | Imperial Laboratories, 500ml, Batch No: 53265, Heat deactivated at 56°C for 35 minutes, stored at -20°C |

| | |
|---|---|
| Forskolin | Calbiochem, Cat No: 344270, 5 mM stock solution made by dissolving 2.0525 mg/ ml in DMSO. Stored at -20°C 50/ 100 µl aliquots |
| BMX, Isobutyl-1-Methylxanthine | Sigma, Cat No: I-5879, Stock solution 50 mM: FW: 222.2, 100 mg dissolved in DMSO. Stored at -20°C as 200 µl aliquots. |
| Insulin, Bovine Pancreas, culture tested | Sigma, Cat No: I 1882, Lot: 57H4626, Stock solution 10 mg/ ml; 10 ml of acidified Water (150 µl of glacial acetic acid added to 15 ml of distilled water, in fume cupboard, 0.2 µm filtered). Dissolved slowly re-filtered with 0.8/0.2 µm. Stored at 4 °C as I ml aliquots. |
| Mouse Anti Human Thy 1.1 | Serotec, Cat NO: MCAP90, 2ml, IgG1 |
| Mouse Anti Mouse/Rat Thy 1.1 | Serotec, Cat No: MCA04G, 0.25mg/ 0.25ml, IgM |
| Penicillin/Streptomycin | Gibco, Cat: 15070-022, Working concentration: 100 iu-100 µg, by adding 2ml to 100 ml of DMEM. |
| Poly-D-Lysine, Lyophilised, MW: 30,000-70,000. | Sigma, Cat No: P7280 |
| Schwann cell growth medium (human) | Rat Schwann cell growth medium (made with high, 4.5g/l, glucose DMEM plus 0.5 mM IBMX, 1.1 ml of stock and 2.5 µg/ml insulin, 28 µl for 100ml. |
| Schwann cell growth medium (neonatal rat) | Cell growth medium plus forskolin, 5µM, GGF 126 ng/ml (of Batch rhGGF2 121195): To 100 ml of cell growth medium 112 µl of froskolin stock 3 µl of GGF added. |
| Trypsin/EDTA 0.05%/0.02% | Gibco, Cat: 45300-019 |

**Human Schwann cell culture**

**Nerve collection**

Peripheral nerve samples are taken from small nerve branches from the sural nerve of the leg. Under sterile conditions, the samples are immediately collected in transfer

medium (OptiMEM) and kept at room temperature for no longer than 24 hours while transferred to the laboratory. On arrival, the nerve samples are washed twice with DMEM.

Using an operating microscope, the perineurium is removed and the nerve branches are divided into fascicles and cut into 1mm pieces. The pieces are washed with DMEM/ HEPES, put in a 25cm$^2$ culture flask and covered with minimal volume human SC growth medium such that the segments remained attached to the flask rather than floating. The culture flask has to be coated with both poly-D-lysine and human laminin. Nerve pieces are incubated with SC culture medium at 37°C for 10 days, with medium changes every 2 days. At the end of the incubation period, the segments are gently removed.

**Digestion and Purification**

*Day 1* The pre-digestion incubation encourage fibroblast migration out of the nerve segments as well as SC mitosis within the nerve. The nerve segments are then digested in a universal container with 2ml of cell culture medium plus 125U/ml of collagenase I and 0.8U/ml of dispase I. The container is kept at 37°C in a flask shaker to allow gentle mixing for 2 hours. If the pieces have not broken down, thus not yet digested, the process is continued for further 30-60 minutes. The resultant mixture is triturated through decreasing calibre needles (19G, 21G and 23G) filtered through a 70µm cell filter and the filter is flushed with 5ml of DMEM. The cell suspension is centrifuged at 800rpm and the pellet re-suspended in 10ml of OptiMEM and plated on a PDL/laminin double coated flask. After 24 hours, most SC have settled and the medium is changed to a human SC growth medium.

Unlike rat SC, human SC cultures do not routinely undergo immuno-purification as the pre-digestion incubation eliminate most of the fibroblasts. However, anti human Thy 1.1 antibodies together with rabbit complement, are sometimes used to eliminate excess fibroblast growth.

*Day 2* The culture flasks are checked for cell attachment and infection. The medium is carefully taken off and the cells are gently washed with DMEM/ HEPES. After washing, 5ml of growth medium containing 10µM cytotoxic agent cytosine-β-D-arabinofuranoside (Arac C) is added and the flasks are returned to the incubator.

*Day 3* The cultures are checked for fibroblast overgrowth and incubation with fresh medium containing with Ara-C is repeated.

*Day 4* The old medium is aspirated, followed by 4 gentle washes of cells with DMEM/HEPES. 5ml of SC growth medium is added to the culture and the flasks are incubated until the SC are confluent, which takes up to 4 days.

*Day 5-8:* *Fibroblast depletion:* This stage is undertaken once SC (mixed with fibroblasts) are sub-confluent. SC are lifted off the flask by adding 0.25% trypsin/ EDTA (2ml for a 25cm$^2$ flask, 3-4ml for a 75cm$^2$ flask). After approximately 5 minutes incubation the cells start to lift off, then the flask is sharply tapped to detach completely all the cells. Following a check under the inverted microscope to ensure complete cell detachment, 5ml of cell growth medium is added to stop the trypsin action. The suspension is centrifuged at 800rmp for 5 minutes. The supernatant is aspirated and the cells are washed again and centrifuged leaving a residual of around 0.1ml. The cells are re-suspended and 500µl of diluted mouse anti rat Thy 1.1 antibody (dil. 1:1000 in DMEM) is added and the mixture is incubated for 10 minutes. 250µl of complement is added and the cell suspension is incubated for further 30 minutes, with occasional mixing. 10ml of cell growth medium is added to the cell suspension and centrifuged at 800rmp for 5 minutes. The cell pellet is re-suspended in 5ml of SC growth medium and incubated in a PDL/laminin coated 25cm$^2$ flask. This procedure eliminates most of the fibroblast contamination. However, on occasion the procedure is repeated if fibroblast contamination became a problem when SC are passaged. Contamination could easily be detected due to the different morphology of the cells. SC are bipolar cells with long processes, while fibroblasts show larger cell bodies with short processes.

When the SC are confluent and ready to split, they assume a characteristic swirl pattern. The medium is aspirated and the cells are lifted off by addition of 0.25% trypsin EDTA. The suspension is transferred to a universal container and centrifuged at 800rmp for 5 minutes.

**Culturing for transplantation**

The SC are washed with DMEM/ HEPES, counted with a haemocytometer and plated on a PDL/laminin double coated flask with SC growth medium at density of 5x10$^5$ cells/ 25cm$^2$.

Human SC grow more slowly than neonatal rat SC and need medium change every day.

To achieve an appropriate cell number for transplantation culturing has to continue for > 2 weeks after purification.

**Neonatal Schwann cell culture**

Sciatic nerves from 20 neonatal Lewis rats were harvested and digested with 1% collagenase I (Worthington Biochemicals) and 0.25% trypsin (Gibco) over 45 minutes. The digestant was triturated through 21G and 23G needles and filtered through a 70 µm cell filter

(Falcon). The filterant was then centrifuged at 800rpm for 5 minutes and cells were resuspended in basic medium: Dulbecco's Minimum Eagles Medium plus Glutamax®, DMEM (Gibco), penicillin, 100 iu/ml and streptomycin 100 g/ml, (Gibco), 10% fetal calf serum, FCS (Imperial Laboratories) and plated on a 25 $cm^2$ poly-D-lysine (Sigma) coated flask and kept at 37°C, 95% humidity, 5% $CO_2$. The following day the medium was changed to basic medium containing 10μM cytosine-β-D-arabinofuranoside (Sigma) and incubated for 38 hours to stop fibroblast growth. The majority of the cells remaining were SC and the medium was changed to SC growth medium i.e. basic medium with the addition of recombinant glial growth factor II 63ng/ml ($Max^{1/2}$ activity, 4.8μg/ml) (Cambridge NeuroScience), and 10μM forskolin (Calbiochem). Once the cells became confluent the final stage of purification was carried out, cells were trypsinised (Gibco) and the suspension was centrifuged at 800rpm for 5 minutes. Cells were resuspended in 1 ml of medium containing mouse anti Thy 1 (di. 1:1000, Serotec, MCA04) incubated at 37°C for 30 minutes, followed by addition of 250μl of rabbit anti mouse complement (Cedarlane) and further incubation of 15 minutes. The cells (around $120x10^5$) were then washed and grown on poly-D-lysine (Sigma) coated flasks, the medium was changed every 48 hours and SC were split, 1 in 3, on confluence (12,13). At the second passage, following purification, the rats SC were transduced.

**Genetic labelling of Schwann cells**

The retroviral vector pMFG *lac*Znls (14) was utilised to introduce modified *E. coli lacZ* marker gene that encodes the β-galactosidase protein and nuclear localisation sequence (nls) into SC. For this purpose the Moloney Murine Leukaemia Virus (MMLV) packaging cell line PT67 (Clontech, USA) was used. This contains the three retroviral structural genes in its genome but lacks the packaging signal which was provided in trans as part of the MFG *lacZ* nls containing the signal and two long terminal repeats (LTR) with *lacZ* gene cloned between them. In this way retrovirus encoding the *lacZ* gene could transduce SC, but the transduced SC could not produce any further retrovirus due to the absence of the retroviral structural genes from their genome. A stable PT67 *lacZ* nls producing clone (titre approximately $10^6$/ml ) was isolated and used throughout this study.

Ten ml of growth medium (high glucose DMEM/ FCS) was put on a confluent layer of PT67 *lacZ* nls producers and kept overnight at 32°C. After incubation the medium containing retroviral particles was filtered through a 0.45μm filter (Nalgene) and added to a flask of 70% confluent SC together with the polyanion polybrene (Sigma) at 8μg/ml. The

transducing medium was left on SC for 4 hours at 32°C, the medium was then taken off and SC growth medium added (15). The SC culture flask was kept at 37°C overnight and this cycle was repeated three times consecutively over 3 days. Transduction rate was 83% prior to transplantation as assessed by 5-bromo-4-chloror-3-indoyl-β-D galactosidase (X-gal) staining to detect β-galactosidase expression (16).

**Implant preparation**

*Schwann cell suspension*

Transduced SC were expanded in culture (no more than 7 passages) and a stock of concentrated SC suspension ($160x10^6$/ml) in fibronectin (Sigma) was prepared and kept at 4°C prior to use, for no longer than 30 minutes.

*Alginate matrix and conduit filling*

A sterile 4% stock solution of ultrapure, low viscosity mannuronic acid alginate (Pronova, Norway) in 0.9% saline solution was prepared. Final matrix was prepared by mixing the stock alginate (50:50 v/v) with fibronectin or SC suspension in fibronectin in order to obtain a final $80x10^6$/ml concentration of SC. PHB conduits (Astra Tech, Sweden) were carefully filled with 30µl of alginate matrix and alginate was set in 0.1M $CaCl_2$ for 2 minutes and filled, conduits were gently rinsed twice in DMEM. Conduits were kept in DMEM at 4°C for no longer than 2 hours prior to implantation.

**Nerve repair model**

Using an operating microscope (Zeiss, Germany), PHB conduits containing SC from Lewis rat origin were grafted in adult male Lewis (for syngeneic) or Dark Agouti (for allogencic) rats (Harlan), average weight of 180g, to bridge a gap of 1 cm in the left sciatic nerve. These two strains of rats have differences in their major histocompatibility complexes (MHC) (17). Conduits without SC were also implanted in a separate group of animals and served as control. Animals were sacrificed as 2, 3 or 6 weeks (n=6 each group) and the conduits were harvested, fixed in Zamboni's fixative and then rinsed in 0.01 M PBS containing 15% (w/v) sucrose and 0,1 % (w/v) sodium azide and kept at 4°C.

**Tissue process and analyses**

The specimens were blocked in OCT compound (Tissue-tek, Sakura, Japan), placing a piece of rat liver next to the proximal end of the nerve to identify the orientation of each sample. The specimens were sectioned longitudinally, 15µm thick, using a cryostat (Bright). Chemical X-gal staining and then fluorescent immunohistochemistry were used to examine labelled SC and regeneration and immunological parameters.

*Axonal regeneration and Schwann cell ingrowth*

Axonal regeneration distance, the amount of new axonal growth as well as that of SC ingrowth into the conduits were quantified after double immunostaining on the same section. After blocking with 1% normal rat and goat sera, the sections were stained using a combination of antisera to S100 for SC identification. (dil 1:1000, rabbit polyclonal, Dako) and pan-neurofilaments (PanNF) for regenerating axons (dil 1:1000, mouse monoclonal, Affinit). Sections were incubated for 2 hours at room temperature, then washed twice with PBS (5 minutes each wash). The secondary fluorescence conjugated antibodies (dil, 1:100, goat anti-rabbit FITC conjugated and goat anti-mouse Cy3 conjugated) were added and the sections incubated for another 1 hour at room temperature. The sections were then washed with PBS (3X5min.) and mounted with Vectorshield fluorescent mountant (Vector Labs Inc,). Axonal regeneration distance was measured from the proximal stump into the graft, using a calibrated microscope graticule (mean of 3 non-consecutive sections per animal). The area, of immunostaining was taken as a measure of the quantity or the axonal regeneration and SC ingrowth into the conduits and was measured across a fixed point to allow comparison between groups, at 3 mm the proximal edge of the conduits and expressed as the percentage of immunostaining per field. A band of images were taken across the whole of the conduit and the overlapping images edited to avoid duplicate measurement..The images were captured with a SPOT digital camera (Diagnostic Images Inc) and analysed using a PC based image analysis software (Image Pro Plus, version 4, Media Cybernetics, USA).

*Transplanted Schwann cell*

X-gal staining was performed on the sections to assess the presence of transduced SC. Sections were examined and scored semi-quantitatively as high, medium, low or zero. In order to assess the involvement of transplanted SC in the regeneration process, chemical X-gal staining was carried out on the same section as immunostaining for PanNF and S100, as above and the sections were examined using a combination of transmitted light and epifluorescent microscopy.

*Schwann cell characterisation*

After blocking with 1% normal rat and goat sera, the following antisera were used for immunostaining on separate but consecutive sections: myelin basic protein (MBP, marker of myelinating SC, mouse monoclonal dil, 1:500, Boehringer), p75 (marker of de-differentiated SC mouse monoclonal dil. 1:40, Boehringer) and neural cell adhesion molecule (NCAM, marker of un-myelinating SC rabbit polyclonal, dil. 1:700, Chemicon). Sections were incubated at 4°C overnight and, then washed twice with PBS (5 minutes each).

The secondary fluorescence conjugated antibodies (dil. 1:100, goat anti-rabbit FITC conjugated and goat anti-mouse Cy3 conjugated) were added and the sections incubated for another 1 hour at room temperature. The sections were then washed with PBS (3X5 min.) and mounted with Vectorshield fluorescent mountant (Vector Labs Inc.). The percentage area of immunostaining in a fixed area was taken as a measure of expression of each SC phenotypic markers. For each section, a digital image was taken at a fixed point, 3 mm from the proximal nerve end and equidistant from the walls of conduit. The percentage area of immunostaining was measured for each field using a PC based image analysis software as above section (mean of 3 non-consecutive sections per animal and per staining).

*Quantification of MHC class I and II*

Immunostaining was carried out as above, with overnight incubation of the sections using the following antisera. MHC I (mouse monoclonal dil 1:20 Serotec, MCA51G, clone OX-18); MHC II (mouse monoclonal dil 1:20 Serotec, MCA46A anti ]-A, clone MRC OX-6). Following immunostaining sections were examined without delay and the intensity of fluorescence was taken as measure of the level of expression of MHC I and II (2 random fields were examined per section and 3 non-consecutive sections per animal and per staining). Digital images were taken at fixed settings to give comparable intensity measurements. Intensity of the field was measured using Image Pro Plus software, the calibration curve was a default straight line setting, which was confirmed with fluorescent beads of standardised luminescence.

*Lymphocyte and macrophage count*

In order to assess the immune response to the conduits the number of B-lymphocytes, T-lymphocytes and macrophages were counted following immunostaining. The following antisera were used with overnight incubation at 4°C: CD2 (for B-lumphocytes, mouse monoclonal dil, 1:30 Serotec), CD45R (for T-lymphocytes mouse monoclonal dil. 1:60, Serotec) and macrophage (mouse monoclonal dil. 1:200, Serotec). Immunostaining for each antibodies was done on separate but consecutive, sections to allow comparison between the different primary antisera (3 sections per sample and antibody). The number of positively stained cells were manually counted across the whole conduits and expressed as the mean number of stained cells per section.

*Statistical analysis*

One way analysis of variance (ANOVA) was performed to assess significant difference between groups, Tukey's test for comparisons between experimental and control groups, using a SigmaStat statistical analysis package (Jandel Corp, USA).

**Summary of results**

An identifiable and pure population of cultured Schwann cells (SC) was obtained. Transduction of *lacZ* genetic label was carried out and a stable population of genetically modified SC was obtained. Transduced SC properties and *lacZ* expression were preserved *in vitro* for 6 months of continuous culture.

Suspension matrix is required for SC transplantation and the suitability of alginate hydrogel was confirmed by *in vitro* tests to support SC proliferation and neurite sprouting in a neuron-glial co-culture. Defects in the rat sciatic nerve injury was bridged using resorbable conduits containing SC. The results showed that the optimal number of SC required to enhance axonal regeneration was $80x10^6$/ml and alginate together with SC further improved regeneration. Following transplantation of *syngeneic* and *allogeneic* SC both improved axonal regeneration distance, but the quantity of regeneration was better and more sustained with *syngeneic* SC.

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## Claims

1. Nerve repair unit comprising a resorbable polymeric support and an alginate matrix containing human Schwann cells.

2. Nerve repair unit according to claim 1, wherein the polymer of the resorbable polymeric support is selected from the group consisting of polyhydroxybutyric acid, polyglycolic acid and polylactic acid.

3. Nerve repair unit according to claim 1, wherein the alginate matrix is an ultrapure, low viscosity mannuronic acid alginate.

4. Nerve repair unit according to claim 1, wherein the resorbable polymeric support is a polyhydroxybutyric acid conduit.

5. Method of producing a nerve repair unit comprising a resorbable polymeric support and an alginate matrix containing human Schwann cells, comprising the steps of mixing an alginate in isotonic saline solution with a human Schwann cell suspension in fibronectin, supplying the mixture to the resorbable polymeric support, setting the alginate matrix with a solution of calcium chloride in human cell culture medium, rinsing with the human cell culture medium and keeping the nerve repair unit in the human cell culture medium until use.

6. Method of producing a nerve repair unit according to claim 5, wherein the polymer of the resorbable polymeric support is selected from the group consisting of polyhydroxybutyric acid, polyglycolic acid and polylactic acid, the alginate is ultrapure, low viscosity mannuronic acid alginate and the human cell culture medium is Dublbecco's Minimum Eagles Medium plus Glutamax® (DMEM).

7. Method of producing a nerve repair unit according to claim 5, wherein the human Schwann cells are produced by transporting a sample of a peripheral nerve of a human patient in a transfer medium at ambient temperature within 24 hours to a competent laboratory, upon arrival washing the sample with a human culture medium, removing the perineurum, dividing the nerve branches into fascicles and cutting them into pieces, washing the pieces with a human culture medium and placing them into a culture flask coated with both poly-D-lysine and human laminin and covering them with a Schwann cell culture medium, followed by incubation at 37°C for up to 10 days, changing the medium every 2 days, and removing the nerve segments for digestion in a container with cell culture medium plus collagenase I and dispase I until the segments have broken down, followed by trituration, filtration, washing and centrifugation, resuspending the resulting pellet in transfer medium

and plating the suspension on a culture flask coated with both poly-D-lysine and human laminin and after 24 hours changing the medium to a Schwann cell culture medium, followed by cultivation for at least 7 days under conditions removing possible fibroblast contamination and changes of culture medium until the Schwann cells are confluent and ready to split, aspirating the medium and suspending the cells in a trypsinized solvent, followed by centrifugation and washing of the pellet, counting the Schwann cells with a haematocytometer and plating them on a culture flask coated with both poly-D-lysine and human laminin and covering them with a Schwann cell culture medium at a density of $5x\ 10^5$ cells/ 25 $cm^2$, culturing the cells for at least 14 days under change of medium every day to produce an appropriate cell number for transplantation to the patient.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 02/02058

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: A61L 31/04, C12N 5/08
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EPODOC, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| E,X | Experimental Neurology, Volume 173, 2002, Afshin Mosahebi et al, "Effect of Allogeic Schwann Cell Transplantation on Peripheral Nerve Regeneration" page 213 - page 223 | 1-6 |
| | -- | |
| Y | GLIA, Volume 34, 2001, Afshin Mosahebi et al, "Retroviral Labeling of Schwann Cells: In Vitro Characterization and In Vivo Transplantation to Improve Peripheral Nerve Regeneration" page 8 - page 17 | 1-6 |
| X | | 7 |
| | -- | |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 19 February 2003 | 20 -02- 2003 |
| Name and mailing address of the ISA/<br>Swedish Patent Office<br>Box 5055, S-102 42 STOCKHOLM<br>Facsimile No. +46 8 666 02 86 | Authorized officer<br>Erika Stenroos/Els<br>Telephone No. +46 8 782 25 00 |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 02/02058

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | Tissue Engineering, Volume 7, No 5, 2001, Ashin Mosahebi et al, "A Novel Use of Alginate Hydrogel as Schwann Cell Matrix" page 525 - page 534 | 1-6 |
| X | -- | 7 |
| A | Biomaterials, Volume 20, 1999, G.R.D. Evans et al, "In vivo evaluation of poly(L-lactic acid) porous conduits for peripheral nerve regeneration" page 1109 - page 1115 | 1-6 |
| | -- | |
| A | British Journal of Plastic Surgery, Volume 52, 1999, A Hazari et al, "A resorbale nerve conduit as an alternative to nerve autograft in nerve gap repair" page 653 - page 657 | 1-6 |
| | -- | |
| | -------- | |

# INTERNATIONAL SEARCH REPORT

ıational application No.
PCT/SE02/02058

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

see next page

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

According to Annex B, Administrative Instructions under the PCT (PCT GAZETTE 1998, June 25) unity of the invention exists only when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding "special technical features". The definition of "special technical features" is stated in PCT rule 13.2 as those technical features that define a contribution, which each of the inventions considered as a whole, makes over the prior art. Consequently this application comprises 2 independent inventions defined as follows:

Invention 1: Claims 1-4, which describes a nerve repair unit consisting of a resorbable polymeric support, an alginate matrix and human Schwann cells. Claim 5 and 6 describes a method for the production of the nerve repair unit. The resorbable polymer is preferably PHB (polyhydroxybutyric acid).

Invention 2: Claim 7 describes a method for preparation of a cell culture.

**PCT** WORLD INTELLECTUAL PROPERTY ORGANIZATION
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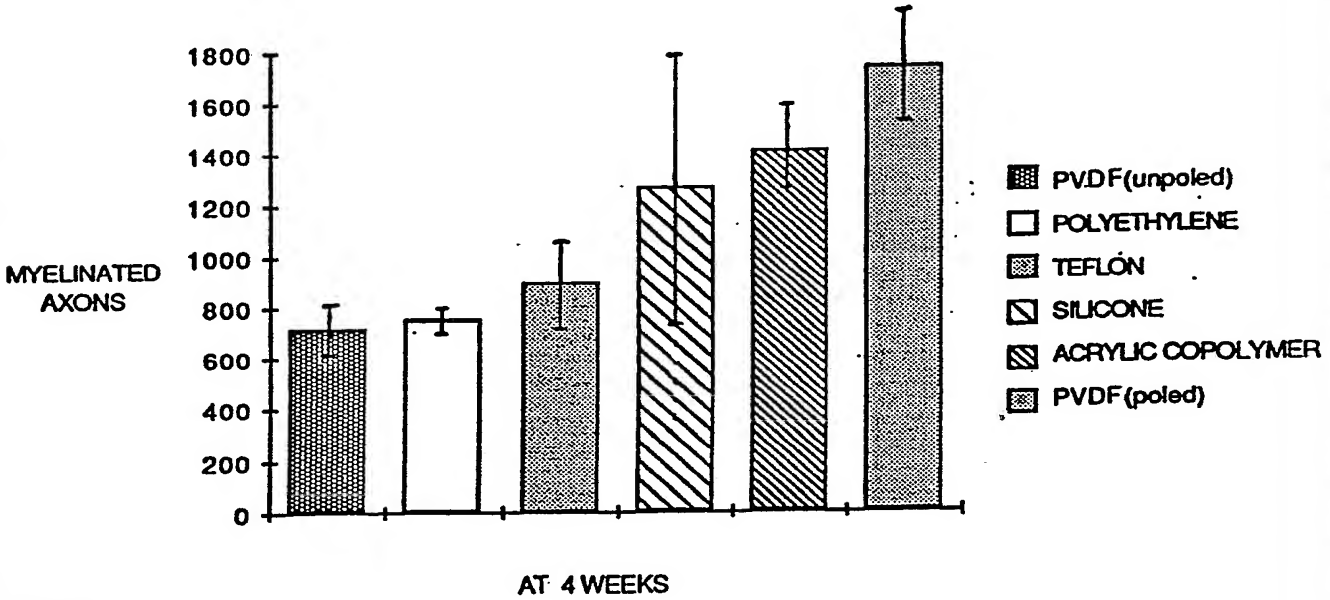
**Published**
*With international search report.*

**(54) Title:** PIEZOLECTRIC NERVE GUIDANCE CHANNELS




**(57) Abstract**

Medical devices and methods employing piezoelectric materials, such as polyvinylidene fluoride (PVDF) and other biocompatible piezoelectric polymers, are disclosed for use as guidance channels in regenerating nerves. The devices can be formed by a tubular piezoelectric membrane adapted to receive the ends of a severed or damaged nerve. The tubular conduit defines a lumen through which axons can regenerate to restore motor and/or sensory functions. The piezoelectric materials generate transient electrical charges upon mechanical deformation which augment the ability of axons to bridge the gap between the proximal and distal nerve stumps.

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# PIEZOELECTRIC NERVE GUIDANCE CHANNELS

## Background of the Invention

The technical field of this invention concerns medical devices useful for the repair of injured nerves and methods for preparing and using such devices for nerve repairs.

The problem of repairing severed nerves is a long-standing one that has plagued surgeons for over a hundred years. Despite advances in microsurgical techniques, a patient's recovery from a serious wound is often limited by a degree of nerve damage which cannot be repaired. The replanting of amputated fingers and limbs is especially limited by poor nerve regeneration.

When a nerve is severed, the functions supplied by that nerve, both motor and sensory, are lost. The nerve cells' appendages (axons) in the distal (the furthest away from the spinal cord) portions of the severed nerve degenerate and die leaving only the sheaths in which they were contained. The axons in the proximal stump that are still connected to the spinal cord or dorsal root ganglion, also suffer some degeneration. The degeneration generally does not proceed to the death of the entire nerve cell bodies. If the injury occurs far enough from the nerve cell bodies,

regeneration will occur. Axonal sprouts will appear from the tip of the regenerating axon. These sprouts grow distally and attempt to reenter the intact neurilemmal sheaths of the distal portion of the severed nerve. If entry is successfully made, axonal growth will continue down these sheaths and function will eventually be restored.

In the conventional approach to nerve repair, an attempt is made to align the cut ends of the fascicles (nerve bundles within the nerve trunk). A similar approach is taken with smaller nerves. In either case, the chief hazard to the successful repair is the trauma produced by the manipulation of the nerve ends and the subsequent suturing to maintain alignment. The trauma appears to stimulate the growth and/or migration of fibroblasts and other scar-forming connective tissue cells. The scar tissue prevents the regenerating axons in the proximal stump from reaching the distal stump to reestablish a nerve charge pathway. The result is a permanent loss of sensory or motor function.

Various attempts have been made over the years to find a replacement for direct (i.e., nerve stump-to-nerve-stump suturing). Much of the research in this field has focused on the use of "channels" or tubular prostheses which permit the cut ends of the nerve to be gently drawn into proximity and secured in place without undue trauma. It is also generally believed that such channels can also prevent, or at least retard, the infiltration of scar-forming connective tissue.

The use of silastic cuffs for peripheral nerve repair was reported by Ducker et al. in Vol. 28, Journal of Neurosurgery, pp. 582-587 (1968). Silicone rubber sheathing for nerve repair was reported by Midgley et al. in Vol. 19, Surgical Forum, pp. 519-528 (1968) and by Lundborg, et al. in Vol. 41, Journal of Neuropathology in Experimental Neurology, pp. 412-422 (1982). The use of bioresorbable polyglactin mesh tubing was reported by Molander et al. in Vol. 5, Muscle & Nerve, pp. 54-58 (1982). The use of semipermeable acrylic copolymer tubes in nerve regeneration was disclosed by Uzman et al. in Vol. 9, Journal of Neuroscience Research, pp. 325-338 (1983). Bioresorbable nerve guidance channels of polyesters and other polymers have been reported by Nyilas et al. in Vol. 29, Transactions Am. Soc. Artif. Internal Organs, pp. 307-313 (1983) and in U.S. Patent 4,534,349 issued to Barrows in 1985.

Despite the indentification of various materials which can serve as nerve guidance channels, the results of research to date have revealed significant shortcomings in such prostheses. Some of the materials identified above have lead to inflammatory reactions in the test animals and have failed to exclude scar tissue formation within the channels. Moreover, the total number of axons, the number of myelinated axons, the thickness of the epineurium, and the fascicular organization of nerves regenerated within guidance channels are all typically less than satisfactory and compare poorly with the original nerve structure of the test animals. Moreover, the loss of sensory or motor function is still the most common outcome of such laboratory experiments.

There exists a need for a better materials and methods for formation of nerve guidance channels. Materials and methods for nerve repair that would minimize surgical trauma, prevent interference with nerve growth by scar tissue, and improve the chances for successful recovery of sensory or motor function, would satisfy a long-felt need in this field.

Summary of the Invention

It has been discovered that the repair of severed or avulsed nerves can be greatly enhanced by the use of piezoelectric materials as nerve guidance channels. Medical devices employing such piezoelectric materials are disclosed for use in regenerating nerves. The devices can be formed by a tubular piezoelectric conduit adapted to receive the ends of a severed or damaged nerve. The tubular membrane defines a lumen through which axons can be regenerated to restore motor and/or sensory functions. The piezoelectric materials generate transient electrical charges upon mechanical deformation which augment the ability of axons to bridge the gap between the proximal and distal stumps.

The term "piezoelectric materials" as used herein is intended to encompass natural and synthetic materials capable of generating electrical charges on their surface when subjected to mechanical strain. The preferred materials are biocompatible, semicrystalline polymers which can be poled during manufacture or prior to use in order to align the polymeric chain segments in a particular orientation and, thereby, establish a predefined dipole moment. The piezoelectric materials of the present invention are preferably poled to establish a charge generation (polarization constant) ranging from about 0.5 to about 35 picoColoumbs per Newton, and, more preferably, from about 1 to about 20 picoColoumbs per Newton.

Piezoelectric materials useful in the present invention include a variety of halogenated polymers, copolymers and polymer blends. The halogenated polymers include polyvinylidene fluoride, polyvinyl fluoride, polyvinyl chloride and derivatives thereof as well as copolymers such as copolymers of the above materials and trifluoroethylene. Non-halogenated piezoelectric polymers which may also be useful in the present invention include collagen, nylon 11, and alpha-helical polypeptides such as polyhydroxybutyrate, poly-γ-benzyl-glutamate and poly-γ-methyl-glutamate. In some applications it may also be possible to use thin piezoelectric ceramics, such as barium titanate, lead titanate or lead zirconate or combinations of such ceramic and polymeric materials.

One particularly preferred piezoelectric material for nerve guidance channels is polyvinylidene fluoride ("PVDF" or "$PVF_2$") especially after it has been poled to impart a high polarization constant. PVDF is a semicrystalline polymer formed by the sequential addition of $(CH_2-CF_2)_n$ repeat units, where n can range from about 2,000 to about 15,000. Crystallographers have described various stable forms or phases of PVDF. The alpha phase, which is generally obtained by cooling the melt at atmospheric pressure, has a monoclinic unit cell with chain segments in antipolar orientation, and thus no net dipole moment.

The beta phase of PVDF, which displays the highest piezoelectric activity, has an orthorhombic unit cell containing two chains with the same orientation, giving it a permanent dipole moment. To display its piezoelectric properties, PVDF must be anisotropic, i.e., its electrical properties must be quantitatively different for mechanical excitation along different axes. In PVDF as well as other semicrystalline polymer films, the isotropy that generally prevails can be altered by molecular orientation, usually induced by mechanical stretching, followed by alignment of the permanent dipoles in a direction perpendicular to the plane of the film by an electric field (a "poling" process).

The use of tubular nerve guidance channels of poled PVDF has been found to surpass all other materials tested to date as guidance channels. When compared with unpoled PVDF, the poled material achieved significantly better results (over twice as many myelinated axons after four weeks) as a nerve guidance material. The success of poled PVDF as a nerve guidance channel material appears to lie in its biocompatibility and high piezoelectric activity. The best results to date have been obtained with tubular PVDF which is poled to generate positive charges on the inner (luminal) surface of the tubes upon mechanical deformation.

The piezoelectric nerve guidance channels of the present invention can also be semipermeable to permit passage of nutrients and metabolites (i.e., having molecular weights of about 100,000 daltons or less) through the channel walls. The permeability can be controlled such that scar-forming cells are excluded from the lumen while growth factors released by the injured nerves are retained within the lumen. Various techniques known in the art, such as the use of degradable derivatives or the formation of copolymers having a biodegradable component can be employed to obtain a satisfactory degree of permeability in use. If the channel is not totally biodegradable over time, it can be formed with longitudinal lines of weakness to facilitate removal from about the regenerated nerve after healing has progressed sufficiently.

Preferably, the membrane wall thickness of the piezoelectric nerve guidance channels of the present invention will range from about 0.05 to about 1.0 millimeters. Similarly, the diameter of lumen can vary from about 0.5 millimeters to about 2 centimeters, depending upon the size of nerve to be repaired.

The invention further encompasses methods for preparing and using piezoelectric nerve guidance channels. In the case of polyvinylidene fluoride (PVDF), the beta phase, which displays the greatest piezoelectric activity, can be obtained by mechanically stretching and annealing alpha phase PVDF. The stretching process orients crystalline unit cells, known as spherulites, with their long axis perpendicular to the direction of elongation. Poling the beta phase PVDF under a high electrical field freezes the random dipoles and creates a permanent, strong dipole moment. The polarity of the electrodes determines the net charge on the outer and luminal surfaces of the tube (i.e., the orientation of the dipoles). Therefore, depending on the poling procedure, tubes which generate upon mechanical deformation either positive or negative charges on their luminal surface can be fabricated. Poling procedures avoiding polymer contact, such as corona discharge, are used to prevent polymer breakdown.

The nerve guidance channels of the present invention are used by locating the severed nerve ends, and selecting an appropriately sized piezoelectric tubular device for the repair, having openings adapted to receive the ends of the severed nerve and a lumen to permit regeneration of the nerve therethrough. The cut ends of the nerve are then gently drawn into tube by manual manipulation or suction, placed in optimal proximity and then secured in position without undue trauma by sutures through the tube, or by a biocompatible adhesive (e.g., fibrin glue) or by frictional engagement with the

tube. The tube is then situated such that muscle contractions and general animal movement induce mechanical deformation and, hence, generation of electrical charges within the lumen. Antibiotics can be administered to the site, and the wound is then closed.

The term "nerve" is used herein to mean both monofascicular and polyfascicular nerves . The same general principals of regeneration with piezoelectric nerve guidance channels are applicable to both.

The invention will next be described in connection with certain preferred embodiments; however, it should be clear that various changes, additions and substractions can be made by those skilled in the art without departing from the spirit or scope of the invention. For example, although the piezoelectric nerve guidance channels described below are generally tubular in shape, it should be clear that various alternative shapes can be employed. The lumens of the guidance channels can be oval or even square in cross-section. The guidance channels can also be constructed from two or more parts which are clamped together to secure the nerve stumps. Moreover, sheet piezoelectric materials can be employed and formed into tube in situ. In such a procedure, the nerve stumps can be placed on top of the sheet and secured thereto by sutures, adhesives or friction. The sheet is then wrapped around the nerve segments and the resulting tube is closed by further sutures, adhesives or friction.

The nerve guidance channels of the present invention can also take advantage of related pyroelectric properties which are often also exhibited by piezoelectric materials. Pyroelectric effects are typically defined as the exhibition of electrical polarization as a result of temperature changes. Thus, the nerve guidance channels of the present invention can also employ temperature changes to create transient electric charges on the inner surface of the lumen.

Various materials can also be used to fill the luminal cavity. For example, the cavity can be filled with physiological saline, laminin, collagen, glycosaminoglycans or nerve growth factors. The cavity can also be seeded with cultured Schwann cells.

Brief Description of the Drawings

FIG. 1 is a comparative graph of the regenerative capabilities (in terms of numbers of myelinated axons) of various piezoelectric and non-piezoelectric nerve guidance materials.

Detailed Description

The invention will next be described in connection with the following examples and comparative experiments.

Young female CD-1 mice (25-30 g) (Charles River Lab., Wilmington, MA) were housed in temperature and humidity-controlled rooms and received food and water ad libitum. The mice were anesthetized with methoxyfluorane and the left sciatic nerve was exposed through an incision along the anterior-medial aspect of the upper thigh. After retraction of the gluteus maximus muscle, a 3-4 mm segment of nerve proximal to the tibio-peroneal bifurcation was resected and discarded.

A series of materials were then tested as nerve guidance channels. The materials were all tubular in shape and 6 mm long. The nerve stumps were anchored 4 mm apart within the tubes using 10-0 nylon sutures placed through holes 1 mm from each channel end. For each material, at least six channels were implanted for a period of four weeks. A further set of control animals underwent nerve resection as described apart, and their section sites were closed without implantation of any guidance material. Aseptic surgical technique was maintained throughout the procedures, which were performed with the aid of an operating microscope.

A variety of non-piezoelectric materials were used as tubular guidance channels for comparison purposes. These non-piezoelectric materials included polyethylene [Clay Adams, Parsippany, NJ], Teflon™ [Gore, Flagstaff AZ], silicone [Silmed, Taunton, MA], and acrylic copolymer (Amicon XD-50 tubing, Lexington, Massachusetts). In addition, non-polarized PVDF was compared with identical PVDF tubing which had undergone poling.

The piezoelectric guidance materials were manufactured from pellets of homopolymeric PVDF (Solef XION, Solvay & Cie, Brussels, Belgium). The pellets were extruded into tubes with an outer diameter (OD) of 2.5 mm and an internal diameter (ID) of 1.85 mm. The extruded tubes were stretched 3.5 times along their axes at a temperature of 110°C and at a rate of 1 cm per minute. This stretching process transform the alpha non-polar crystalline phase into the beta polar crystalline phase. The tubes were then annealed by maintaining the tension on the tubes for 3 hours at 110°C. The final OD and ID were 1.25 mm and 0.87 mm, respectively.

Some tubes were then cut and poled under an electric field to permanently orient the molecular dipoles of the beta phase. A thin wire inserted in the lumen of the stretched PVDF tubes served as an inner electrode and a circumferentially regularly oriented array of steel needles served as the outer electrode. The outer electrodes were connected to the positive output of a voltage supply (Model 205-30P, Bertran Associates Inc., Syosset, NY), and the inner electrode was grounded. The voltage was increased gradually over 2 hours until it reached 21kv and was then maintained for 12 hours. A second set of tubes was poled by connecting the positive output of the voltage supply to the inner electrode and grounding the outer electrode. In both cases, this poling procedure resulted in the generation of surface electrical charges upon mechanical deformation of the tube. Electrical charge distribution was dependent on the local mechanical strain on the tube; the pattern of electrical charges is opposite in the tubes prepared with reversed polarity.

In order to determine the piezoelectric activity of the poled tubes, their outer surface was coated with a thin layer of silver paint and the inner electrode was repositioned. A vertical deflection of 1 mm was induced in the center of each tube by a rotating cam connected to a DC micromotor.

The average charge generated by the tubes poled with positive or negative external electrodes was 200-300 pC. These measurements translated into polarization constants ranging from about 10 to about 15 picoColumbs per Newton for the poled PVDF tubes. Unpoled tubes did not generate a detectable charge upon deformation.

Both poled and unpoled PVDF tubes were washed in acetone, rinsed several times with saline, and cleaned ultrasonically before being sterilized in an ethylene oxide gas chamber at 40°C.

At retrieval time, the animals were deeply anesthetized and perfused transcardially with 5 ml of phosphate-buffered saline (PBS) followed by 10 ml of a fixative containing 3.0% paraformaldehyde and 2.5% glutaraldehyde in PBS at pH 7.4. The operative site was reopened and the guidance channel and segments of the native nerve at either channel end were removed. The specimens were then post-fixed in a 1% osmium tetroxide solution, dehydrated and embedded in Spurr resin. Transverse sections taken at the midpoint of the guidance channel were cut on a Sorvall MT-5000 microtome. The sections (1 micron thick) were stained with toluidine blue. Whole mounts of nerve were displayed on a video monitor through a Zeiss IM35 microscope. Nerve cable cross-sectional area and the number of myelinated axons were determined with the aid of a graphic tablet at a final magnification of 630x. The Wilcoxon Rank-sum test was used to assess statistical differences ($p < 0.05$) between the various populations. All values are presented as means ± standard error of the mean.

The results of the comparative studies are shown in graphic form in FIG. 1. The number of myelinated axons found upon reexposure after four weeks for each of the tested guidance materials is shown. Peripheral nerve regeneration was dramatically enhanced by the use of piezoelectric guidance channels which generated either transient positive or negative charges on their inner surface. Nerves regenerated in poled PVDF tubes contained significantly more myelinated axons and displayed more normal morphological characteristics than nerves regenerated in unpoled tubes. When compared to all other materials tested, the poled PVDF tubes contained the highest number of myelinated axons at four weeks, and the regenerated axons displayed greater diameter and myelin sheath thickness.

Upon reexposure, all retrieved guidance channels were covered by a thin tissue layer which did not reduce the translucence of the PVDF tubes. A cable bridging the nerve stumps was observed in all implanted tubes. All cables were surrounded by an a cellular gel and were free from attachment to the guidance channel wall. In stark contrast, mice with no guidance channel showed complete nerve degeneration.

Nerve cables regenerated in poled and unpoled PVDF tubes differed considerably with respect to their cross-sectional area, relative tissue composition and number of myelinated axons. The cross-sectional area of the regenerated cables at the midpoint of the poled PVDF guidance channels were significantly larger than those regenerated in unpoled PVDF channels ($4.33 \pm 1.25$ versus $2.35 \pm 1.17$ $mm^2 \times 10^{-2}$; $p < 0.05$). The cables were delineated by an epineurium composed mainly of fibroblasts and collagen fibrils which surrounded numerous fascicles containing myelinated and nonmyelinated axons and Schwann cells. The relative percentage of fascicle area was significantly greater in poled tubes whereas the relative percentage of epineurial tissue was significantly smaller (Table 1). Although the relative area of blood vessels was higher in poled tubes, the difference was not statistically significant (Table 1). Most importantly, the nerves regenerated in poled PVDF tubes contained significantly more myelinated axons than those in unpoled PVDF tubes ($1,742 \pm 352$ versus $778 \pm 328$; $p <$ than 0.005).

TABLE 1. COMPARISON OF POLED AND UNPOLED PVDF NERVE GUIDANCE CHANNELS

| | Poled | Unpoled |
|---|---|---|
| Myelinated Axons | 1,742 ± 352 | 778 ± 328 |
| Fascicular Area* | 71.2 ± 3.9 | 65.2 ± 5.1 |
| Epineurial Area* | 19.0 ± 4.0 | 28.0 ± 6.0 |
| Blood vessel Area* | 9.8 ± 3.4 | 6.8 ± 3.2 |

*Relative surface area of the different nerve cable components in percent.

Claims

1. A medical device for use in regenerating a severed nerve, the device comprising a tubular piezoelectric membrane have openings adapted to receive the ends of the severed nerve and a lumen to permit regeneration of said nerve.

2. The device of claim 1 wherein the piezoelectric material has a polarization constant ranging from about 0.5 to about 35 picoColoumbs per Newton.

3. The device of claim 1 wherein the piezoelectric material has a polarization constant ranging from about 1 to about 20 picoColoumbs per Newton.

4. The device of claim 1 wherein the piezoelectric material comprises a material selected from the group of polyvinylidene fluoride, polyvinyl fluoride, polyvinyl chloride, collagen, nylon 11, polyhydroxybutyrate, poly-γ-benzyl-glutamate, poly-γ-methyl-glutamate, copolymers of trifluoroethylene and such polymers, and derivatives of such polymers.

5. The device of claim 1 wherein the piezoelectric material is polyvinylidene fluoride.

6. The device of claim 5 wherein the polyvinylidene fluoride material has a chain length of about 2,000 to about 15,000 repeat units.

7. The device of claim 5 wherein the polyvinylidene fluoride material exhibits a orthorhombic unit cell structure containing two chains with the same orientation and, consequently, a permanent dipole moment.

8. The device of claim 7 where the polyvinylidene fluoride material further exhibits an alignment of the permanent dipoles and a polarization constant of about 1 to about 20 picoColoumbs per Newton.

9. The device of claim 1 wherein the thickness of the membrane ranges from about 0.05 to about 1.0 millimeter.

10. The device of claim 1 wherein the lumen has a diameter ranging from about 0.5 millimeters to about 2 centimeters.

11. The device of claim 1 wherein the membrane is permeable to solutes having a molecular weight of about 100,000 daltons or less.

12. The device of claim 1 wherein the membrane is impermeable to fibroblasts and other scar-forming connective tissue cells.

13. The device of claim 1 wherein the membrane is polarized such that a positive charge is generated at the inner membrane surface upon mechanical deformation.

14. The device of claim 1 wherein the membrane is polarized such that a negative charge is generated at the inner membrane surface upon mechanical deformation.

15. A method for repairing a severed nerve, the method comprising

providing a protective, tubular piezoelectric membrane device having openings adapted to receive the ends of the severed nerve and a lumen to permit regeneration;

placing the severed nerve ends in proximity to each other within the lumen; and

securing the nerve ends to the device.

16. The method of claim 15 wherein the step of securing the nerve ends further includes suturing the nerve ends to the membrane.

17. The method of claim 15 wherein the step of securing the nerve ends further includes securing the nerve ends with an adhesive.

18. The method of claim 15 wherein the nerve ends are secured by friction.

19. The method of claim 15 wherein the method further includes filling the lumen with saline.

20. The method of claim 15 wherein the method further includes filling the lumen with a matrix material selected from the group of laminin, collagen and glycosaminoglycan.

21. The method of claim 15 wherein the method further includes seeding the lumen with a nerve growth factors.

22. The method of claim 15 wherein the method further includes seeding the lumen with Schwann cells.

23. The method of claim 15 wherein the method further comprises the step of allowing the device to undergo biodegration in vivo.

24. The method of claim 15 wherein the method further comprises the step of splitting the tube along lines of weakness and then removing it from the nerve ends after they have joined.

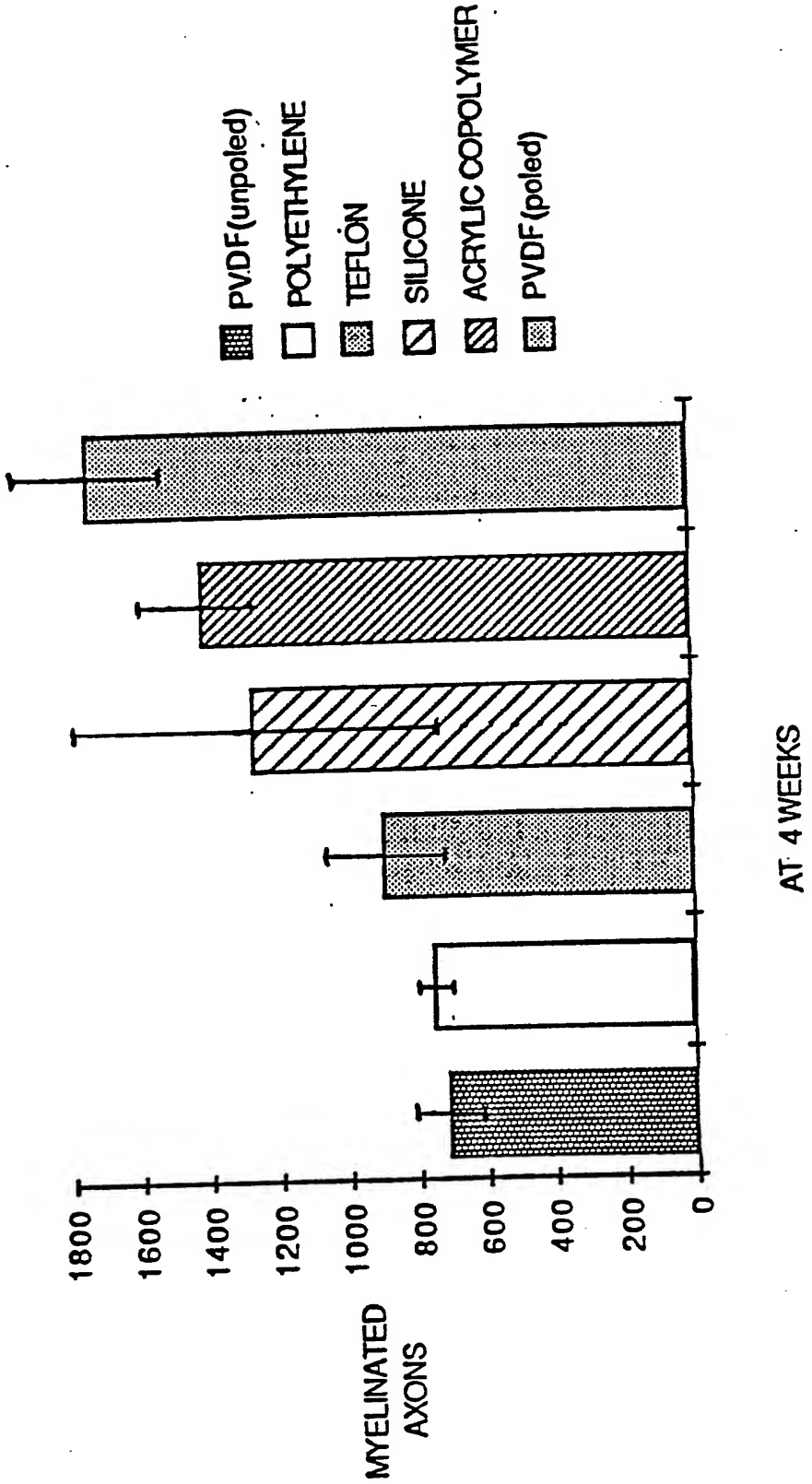
PVDF(unpoled)
POLYETHYLENE
TEFLON
SILICONE
ACRYLIC COPOLYMER
PVDF(poled)
MYELINATED AXONS
1800
1600
1400
1200
1000
800
600
400
200
0
AT 4 WEEKS

FIG. 1

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/00693

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (4): A61B 17/04; C08F 14/18
U.S. Cl. 128/334R; 310/800; 526/255

## II. FIELDS SEARCHED

Minimum Documentation Searched [7]

| Classification System | Classification Symbols |
|---|---|
| U.S. | 128/334R; 310/800; 526/255 |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

## III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

| Category * | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No. [13] |
|---|---|---|
| X<br>Y | American Society For Artificial Internal Organs Journal, Volume 6, No. 1, published 1983 (J.B. Lippincott Co., U.S.), "The Electromechanical Connection: Piezoelectric Polymers in Artificial Organs", see pages 1-11. | 1, 4, 5, 6, 7<br>2, 3, 8-14 |
| A | US, A, 4,074,366 (CAPOZZA) 02 February 1978 See the entire document. | 1 |
| Y | Journal of Neuropathology and Experimental Neurology, Volume 41, No. 4, published July 1982, "In Vivo Regeneration of Cut Nerves Encased in Silicone Tubes", see pages 412-422. | 15-24 |
| Y | Journal of Neuroscience Research, Volume 9, published 1983, "Mouse Sciatic Nerve Regeneration Through Semipermeable Tubes: A Quantitative Model", see pages 325-338. | 15-24 |

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 10 May 1988 | 08 JUN 1988 |
| International Searching Authority | Signature of Authorized Officer |
| ISA/US | Gary Jackson<br>Gary Jackson |

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No |
|---|---|---|
| A | US, A, 3,925,339 (ISHII) 09 December 1975<br>See the entire document. | 1 |
| Y | Mechanisms Of Growth Control, Chapter II, Fukada, Echi, Springfield, MD, pp. 192-210, see entire chapter.(1981) | 15, 16 |
| Y | US, A, 4,534,349 (BARROWS) 13 August 1985<br>See the entire document. | 15, 16, 18 |
| Y | US, A, 3,916,905 (KUHN) 04 November 1975<br>See column 2,lines 48-52. | 17 |
| Y | US, A, 3,833,002 (PALMA) 03 September 1974<br>See column 3, lines 47-52. | 19-21 |
| Y | US, A, 3,786,817 (PALMA) 22 January 1974<br>See columns 2 and 4. | 19-21, 24 |

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

| | | |
|---|---|---|
| Y | Journal of Plastic and Reconstructive Surgery, Volume 74, No. 2, published 08 August 1983, "Nerve Regeneration through Synthetic Biodegradable Nerve Guides: Regulation by the Target Organ", see pages 173-181. | 22-23 |
| A, P | US, A, 4,668,449 (SONI) 26 May 1987 See the entire document. | 1 |
| A | US, A, 4,268,653 (UCHIDOI) 19 May 1981 See the entire document. | 1 |

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

| | | |
|---|---|---|
| Y | Brain Research, Volume 342, published 1985, (Elsevier Science Publishers B.V.) "An In Vivo Model to Quantify Motor and Sensory Peripheral Nerve Regeneration Using Bioresorbable Nerve Guide Tubes" See pages 307-315 | 19-22 |

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

I. Claims 1-14, drawn to a medical device classified in classified in Class 623, subclass 12.

II. Claims 15-24, drawn to a method of repairing a nerve, classified in Class 128, subclass 334.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. Telephone Practice

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.